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Appln. Trans.
PATENT

UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional
applications under 37 CFR 1.53(b))

Attorney Docket No. AP32573-A-A / 072396.0203

First Named Inventor Robbins et al.

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Assistant Commissioner for Patents
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Sir:

Enclosed herewith for filing is a patent application of Robbins et al. entitled IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

which includes:

<input checked="" type="checkbox"/> Specification	<u>70</u> Total Pages
<input checked="" type="checkbox"/> Claims	<u>22</u> Total Pages
<input checked="" type="checkbox"/> Abstract	<u>1</u> Total Pages
<input checked="" type="checkbox"/> Drawing(s)	<u>38</u> Total Sheets
<u> </u> formal	
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☐ Combined Declaration and Power of Attorney Total Pages

☐ Newly executed (original or copy)

☐ Copy from a prior application
(for continuation/divisional only - must be filed to avoid surcharge for late filing)

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☐ Continuation ☐ Divisional ☐ Continuation-In-Part (CIP)
of prior application No.

☐ Amend the specification by inserting, before the first line, the following sentence:

"This is a ☐ continuation ☐ divisional ☐ continuation-in-part
of copending application Serial No. filed ."

Attorney Docket No. AP32573-A-A / 072396.0203

- ☐ An Assignment of the invention to University of Pittsburgh of the Commonwealth System of Higher Ed.
☐ is attached. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
☒ will follow.
☐ has been filed in the prior application

- ☒ Small Entity Statement(s) **ENCLOSED**.
☐ Small Entity Statement filed in prior application. Status still proper and desired.

- ☐ Information Disclosure Statement (IDS) PTO-1449
☐ Copies of IDS Citations.

☐ Preliminary Amendment

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☐ Cancel in this application original claims _ of the prior application before calculating the filing fee.

The filing fee has been calculated as shown below:

FOR	(Col. 1) No. Filed	(Col. 2) No. Extra	Small Entity Rate	Fee	OR	Other Than A Small Entity Rate	Fee
Basic Fee				\$345.00			
Total Claims	59	-20 = 39	x 9 =	\$351.00	x 18 =		\$0.00
Ind. Claims	11	-3 = 8	x 39 =	\$312.00	x 78 =		\$0.00
Multiple Dependent Claim		4	+ 130 =	\$130.00	+ 260 =		
			Total	<u>\$1,138.00</u>			<u>\$0.00</u>

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Total Fees Enclosed \$1,138.00

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Appln. Trans.
PATENT

Attorney Docket No. AP32573-A-A / 072396.0203

Priority

☐ Priority of application Country , Appln. No. filed is claimed under 35 U.S.C. 119.

☐ Certified Copy of Priority Document(s) Country , Appln No. , filed .

☐ is/are attached ☐ will follow ☐ has been filed in the parent application S/N .

☒ Priority of U.S. Provisional Application No. 60/151,980 filed September 1, 1999 and U.S. Provisional Application No. 60/188,944 filed March 13, 2000 is claimed under 35 U.S.C. 119(e).

☒ The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16, 1.17, and 1.21(h) associated with this communication or credit any overpayment to Deposit Account No. 02-4377. Two copies of this sheet are enclosed.

BAKER BOTTS L.L.P.

By

Rochelle K. Seide
Rochelle K. Seide

PTO Registration No. 32,300

Enclosures

005121-03100
007680-237E5960

Applicant or Patentee: Robbins et al.Serial or Patent No.: To be assigned Filed or Issued: August 31, 2000For: IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOMPLASMIC AND/OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION University of Pittsburgh of the Commonwealth System of Higher EducationADDRESS OF ORGANIZATION 200 Gardner Steel Conference Center, Pittsburgh, PA 15260

TYPE OF ORGANIZATION _____

- ☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE [26 USC 501(a) and 501(c)(3)]
☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE ____)
(CITATION OF STATUTE ____)
☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE ____)
(CITATION OF STATUTE ____)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled _____ by inventor(s) _____ described in

- ☒ the specification filed herewith
☐ Application Serial No. __, filed __.
☐ Patent No. __, issued __.

I hereby declare that the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(c) or by any concern which would not qualify as a

Attorney's Docket No. _____

small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *Note: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

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ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

NAME _____

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Frances J. ConnellTITLE IN ORGANIZATION Director, Office of Technology Transfer and Intellectual PropertyADDRESS OF PERSON SIGNING 200 Gardner Steel Conference Center, Pittsburgh, PA 15260SIGNATURE Frances J. Connell DATE 08-31-00

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1 TO ALL WHOM IT MAY CONCERN:

6 Be it known that WE, Paul D. Robbins; Zhibao Mi; Raymond Frizzell; Joseph C. Glorioso; and Andrea Gambotto, citizens of the United States of America, China the United States of America, the United States of America, and Italy respectively, whose post office addresses are 191 Main Entrance Drive, Mt. Lebanon, Pennsylvania 15228; 221 Buchanan Place, Apt. C2, Pittsburgh, Pennsylvania 15228; 5304 Forbes Ave., Pittsburgh, Pennsylvania 15217; 202 Scarborough Dr. Cheswick, Pennsylvania 15024; 1250 South Negley Ave. Pittsburgh, Pennsylvania 15217, respectively, have invented an improvement in

1 IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND /OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

16 of which the following is a

SPECIFICATION

This invention was made in part with support from the National Institutes of Health under grant number AR-6-2225. Therefore, the United States Government has certain rights in the invention.

21

This application claims priority under 35 U.S.C. 119(e) to U.S. Provisional Application Serial Number 60/151,980, filed September 1, 1999 and U.S. Provisional Application Serial Number 60/188,944, filed March 13, 2000.

FIELD OF THE INVENTION

The present invention relates to peptides which facilitate the delivery, uptake and transport of proteins, DNA and viruses into the cytoplasm and/or nuclei of cells as well as methods for the identification of such peptides.

BACKGROUND OF INVENTION

The ability to deliver nucleic acids, amino acids, small molecules, viruses, etc. (hereafter referred to collectively as "cargo") to specific cell types is useful for various applications in oncology, developmental biology, gene therapy and in the general understanding of the mode of operation of particular proteins, nucleic acids and small molecules in a model system. There are a number of viral and nonviral delivery systems which have been developed, including vectors derived from human adenoviruses, herpes simplex viruses, adeno-associated viruses, retroviruses (Mulligan, 1993, *Science* 260:926-932; Berns and Giraud, 1995, *Ann. N.Y. Acad. Sci.* 772:95-104; Smith, 1995, *Ann. Rev. Microbiol.* 49:807-838) and others. Nonviral delivery systems include liposomes and conjugates of plasmid and/or DNA with agents designed to facilitate recognition of specific cell surface receptors and protect the newly introduced intracellular DNA from degradation (Wu and Wu, 1987, *J. Biol. Chem* 262:4429-4432; Curiel et al., 1991, *Proc. Natl. Acad. Sci.* 88:8850-8854; Wagner et al., 1992, *Proc. Natl. Acad. Sci.* 89:6099-6103; Zatloukal et al. 1993, *Gene* 135:199-207; Douglas et al., 1996, *Bio/Technology* 14:1574-1578; Zeigler et al., 1996, *Transplantation* 61:812-817; Felgner, 1997, *Sci. Am.* 276:102-106).

The cell recognition specificity of viruses and viral vectors is generally very high,

1 and their ability to transfer genetic material into a target cell makes them particularly attractive
 candidates for the delivery of cargo to a target cell. However, there are potential risks and
 limitations associated with the use of viral vectors for the delivery of cargo, such as the
 possibility of integration into a host genome by retroviral vectors, and adverse host reactions
 (e.g. immunological reactions) against other viral vectors, such as adenovirus. See, e.g., Yang et
 6 al., 1995, *J. Virol.* 69:2004-2015.

Receptor-mediated endocytosis is widely exploited in experimental systems as a
 natural pathway for the targeted delivery of cargo. Endocytic pathways have been used for
 selective delivery of therapeutic and other biologically active agents to specific cells and to
 particular intracellular compartments. See generally, Shen et al., 1992, *Adv. Drug Deliv. Rev.*,
8:93-113; Kato and Sugiyama, 1997, *Crit. Rev. Ther. Drug Carrier Syst.* 14:287-331. In these
 systems, ligands to cell-specific receptors are either conjugated to cargo, for example,
 macromolecules (Vitetta et al., 1993, *Immunol. Today* 14:252-259; Kuzel and Rosen, 1994, *Curr.*
Opin. Oncol. 6:622-626), liposomes (Kirpotin et al., 1997, *Biochemistry* 36:66-75; Spragg et al.,
 1997, *Proc. Natl. Acad. Sci. USA* 94:8795-8800), radioisotopes or toxins (Fitzgerald, 1996,
 16 *Semin. Cancer Biol.* 7:87-95) and synthetic gene complexes (Wu and Wu, 1993, *Adv. Drug*
Deliv. Rev. 12:159-167), or expressed on the surface of viral transfection vehicles (Kozarsky and
 Wilson, 1993, *Curr. Opin. Genet. Dev.* 3:49-503; Wickham et al., *Gene Ther.* 2:750-756).

Early in the development of receptor-mediated delivery strategies, a ligand was
 used, together with a polycation (such as polylysine) for the targeting of a condensed DNA to a
 21 cell where the ligand was specific for a particular cell surface receptor. See Wu and Wu, 1987, *J.*

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1 translate into an inability to transfer sufficient quantities to a specific target cell.

One approach to improving the ability to transfer sufficient quantities of cargo to specific cells is to identify novel cell-targeting ligands, which increase the rate and specificity for the transport of molecules. The first protein discovered having such transduction properties was the HIV transactivator protein, TAT. See Green & Lowenstein, *Cell*, 55:1179-1188 (1988); Frankel & Pabo, *Cell* 55:1189-1193 (1988). Subsequently, an 11 amino acid transduction domain in TAT (TAT-PTD) responsible for the observed transduction properties was identified, based on its high basic residue content. See Fawell et al., *Proc. Natl. Acad. Sci. USA* 91:664-668 (1994). It has been shown that fusion protein constructs containing TAT-PTD are capable of delivering proteins to a wide spectrum of cell types both *in vitro* and *in vivo*. See Nagahara et al., *Nat. Med.* 4:1449-52 (1998); Vives et al., *J. Biol. Chem.* 272:16010-17 (1997); Shwarze et al., *Science* 285:1569-72 (1999); Vocero-Akbani et al., *Nat. Med.* 5:29-33 (1999); Moy et al., *Mol. Biotechnol.* 6:105-13 (1996). It is not known however if TAT-PTD will be effective in all cells and with all fusion constructs. It is possible that TAT-PTD will elicit an immune response in subjects to which it is administered. See Schwarze & Dowdy, *TiPS* 21:45-48. Furthermore, the half-life of TAT-PTD may vary in different cells and subjects which could also adversely effect its transduction efficiency. See Schwarze & Dowdy, *TiPS* 21:45-48.

In addition, a class of peptides, called penetratins, which have translocating properties and are capable of carrying hydrophilic compounds across the plasma membrane have recently been identified. For example, Derossi et al., 1998, *Trends in Cell Biology* 8:84-87, have isolated a 16 residue peptide (called penetratin-1, Ant PTD, or AntP) possessing translocation

1 properties, corresponding to amino acids 43-58 of the homeodomain of ANTENNAPEDIA, a
2 *Drosophila* transcription factor which is internalized by cells in culture. The 16 residue peptide
3 has translocation properties equivalent to those of the full length homeodomain. Derossi et al.
4 have shown the ability of the 16 residue peptide to intracellularly deliver oligonucleotides and
5 oligopeptides attached thereto. However, this method is limited in that oligonucleotides greater
6 than 55 bases long and oligopeptides greater than 100 amino acids long were not shown to be
7 efficiently delivered. Additionally, the peptide-oligonucleotide and peptide-oligopeptide hybrids
8 may be insoluble. Furthermore, delivery was inhibited by the release by cells (particularly dying
9 cells) of DNA into the extracellular matrix which binds to the peptide and inhibits
10 internalization. These peptides are also susceptible to the problems of specificity and affinity for
11 particular cell types.

12 Similarly, Villaverde et al. have isolated a short peptide which contains the cell
13 attachment motif of foot and mouth disease virus (FMDV). Villaverde et al., 1998,
14 *Biotechnology and Bioengineering* 59:294-301. This peptide targets a specific receptor and the
15 mechanism of import is also receptor mediated. Villaverde et al. demonstrated that the
16 attachment of the FMDV peptide to β -galactosidase (β gal) facilitated the uptake of β gal into
17 cells *in vitro*. The attachment of the peptide was either at the n-terminus of β gal or at an internal
18 loop of β gal. Internal attachment provided superior internalization of β gal, and attachment of
19 multiple copies further increased the amount of internalization. This peptide demonstrated
20 varying affinity for different cell lines and therefore is likely to work efficiently with only
21 particular target cells.

1 Elliot & O'Hare (*Cell*188:223-233 (1997)) have shown that VP-22, a 38kDa
tegument protein from herpes simplex virus type 1 (HSV-1) also possesses the ability to
transduce attached molecules across cell membranes and that residues 267-300 of VP-22 are
required, but may not be sufficient, for transduction. Since the region responsible for
transduction has not yet been identified, current approaches using VP-22 have been directed to
6 fusing the entire VP-22 protein to a molecule to facilitate the transduction of that molecule. This
has several disadvantages including a greater likelihood that the fusion protein (1) will be more
readily degraded in cells, (2) will be harder to produce due to solubility problems, and (3) will
elicit an immune response in a subject. In addition, there is little data about the efficiency of
transduction using VP-22 linked to another molecule. See Schwarze & Dowdy, *TiPS* 21:45-48.

Therefore, there is a need for a simplified, improved delivery means for delivering
cargo, such as polypeptides, polynucleotides, small molecules, plasmids and viruses to cells
which demonstrates high efficiency transfer of the cargo to a wide variety of cell types. There is
also a need for a method for isolating such improved means (*e.g.* peptides) for the delivery of
cargo into a wide variety of cell types at high efficiency.

16 SUMMARY OF THE INVENTION

The present invention relates to internalizing peptides which are capable of
facilitating the delivery, uptake and, where desired, nuclear and/or cytoplasmic transport of cargo
(*e.g.* polynucleotides, polypeptides, small molecules, virus, modified virus, plasmid, etc.) into a
target cell. The internalizing peptides of the invention are isolated according to their ability to
21 efficiently internalize and deliver cargo into a wide variety of cell types. The peptides of the

1 invention can facilitate transport from the extracellular milieu to the cytoplasm and/or nucleus in
a cell both *in vivo* and *in vitro*.

6 The peptides of the present invention are useful, *inter alia*, for (1) facilitating the
uptake of cargo in a target cell; (2) inducing apoptosis in cells (*e.g.*, arthritic cells, tumor cells,
etc); (3) expanding a population of stem cells; (4) expanding a population of differentiated cells;
(5) stimulating the differentiation of a population of stem cells; (6) facilitating the integration of
AAV DNA into the genome of a cell; (7) facilitating the uptake into a cell, secretion from said
cell and subsequent reuptake into a neighboring cell of a protein; (8) facilitating the growth of
defective viruses in culture; (9) stimulating the immune response in a subject; (10) facilitating
uptake of any GST fusion protein into a cell; (11) eliciting an immune response in a subject; and
(12) facilitating the delivery of immunogens (*e.g.* vaccines), whether protein based, DNA based,
vector based or viral based.

16 The present invention also relates to a method for identifying internalizing
peptides which are capable of facilitating the uptake and cytoplasmic and/or nuclear transport of
cargo into a target cell. The method comprises (a) incubating a target cell with a peptide display
library; (b) isolating internalized peptides presented by said peptide display library from the cells
and identifying said internalized peptides; (c) linking said peptides to cargo; (d) incubating said
peptide-cargo complex with a target cell; and (e) determining the ability of said peptide to
facilitate the uptake and, where desired, cytoplasmic and/or nuclear localization of said cargo
into said target cell.

21 In addition, the present invention provides for immunogens comprising an

1 internalizing peptide of the present invention linked to cargo and for a method of eliciting an
 immune response in a subject comprising delivering the peptide/cargo complex (*i.e.* the
 immunogen) of the present invention to target cells of the subject. In one preferred embodiment
 of the invention, the immunogen is a vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

6 Figure 1 A&B: (A) shows the ability of peptides 1, 2, 3, 4, 5, 6 of the invention,
 antennapedia peptide (Ant-PTD), TAT-PTD and a random peptide (SEQ ID NO:1, SEQ ID
 NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:19, SEQ ID
 NO:21 and SEQ ID NO:20 respectively) to facilitate the uptake of β gal, when linked through a
 biotin-streptavidin bridge, into HIG-82 cells. (B) Shows the ability of peptides 2, 3, 4, 5,
 antennapedia peptide, TAT-PTD, a random peptide (SEQ ID NO:2, SEQ ID NO:3, SEQ ID
 NO:4, SEQ ID NO:5, SEQ ID NO:19, SEQ ID NO:21 and SEQ ID NO:20 respectively) and β -
 gal alone to facilitate the uptake of β gal into HIG-82 cells at differing concentrations (1:1 = 150
 nM of β -gal).

Figure 2 A-D: Figure 2 A and 2C (low and high magnification respectively)
 16 shows the ability of peptide 1 (SEQ ID NO:1) to facilitate the uptake of β -gal into rabbit synovial
 cells and Figure 2B and 2D (low and high magnification respectively) shows the ability of
 peptide 3 (SEQ ID NO:3) to facilitate the uptake of β -gal into rabbit synovial cells.

Figure 3 A-D: Figure 3A and 3B (high and low magnification respectively)
 shows the ability of peptide 5 (SEQ ID NO:5) to facilitate the uptake of β -gal in human synovial
 21 cells and Figure 3C and 3D (high and low magnification respectively) shows the ability of

1 peptide 1 (SEQ ID NO:1) to facilitate the uptake of β -gal in human synovial cells.

Figure 4 A & B: (A) shows the ability of peptides 2, 3, 4, 5, TAT-PTD, antennapedia peptide and a random peptide (SEQ ID Nos:2, 3, 4, 5, 21, 19 and 20 respectively), to facilitate the uptake of β -gal in rabbit synovial lining; (B) histology of rabbit synovial lining using eosin counter-stain.

6 Figure 5 shows the ability of various peptides (peptides 1-6; SEQ ID Nos:1-6 respectively) to compete for binding in Hig-82 cells.

Figure 6 A-I: shows the ability of peptide 5 (SEQ ID NO:5) to facilitate the uptake of β -gal in (A) HIG-82 cells; (B) rabbit primary synovial cells; (C) human primary synovial cells; (D) primary human airway epithelial cells HBE 144; (E) polarized canine kidney cells MDCK; (F) human islet primary cells; (G) murine myoblast cells C2C12; (H) murine fibrosarcoma tumor cells MCA205; and (I) NIH3T3 cells.

Figure 7 shows the ability of peptide 3 (SEQ ID NO:3) and peptide 5 (SEQ ID NO:5) to internalize Cy3 labeled-M13 phage when said peptides are expressed on the surface of the phage.

16 Figure 8 shows the ability of peptide 3 (SEQ ID NO:3), peptide 5 (SEQ ID NO:5) and the antennapedia peptide (P.P) (SEQ ID NO:19) to facilitate the uptake of β -gal when linked thereto into tumor cells *in vivo* following intra-tumoral injection.

21 Figure 9 A-G: (A) schematic drawing of the vector construct for eGFP fusion protein; (B & C) shows the ability of peptide 5 to internalize eGFP in human islets at low magnification (B is a photomicrograph of the histologically stained cells and C shows the

1 flourescent detection of eGFP); (D & E) shows the ability of peptide 5 to internalize eGFP in
human islets at high magnification (D is a photomicrograph of the histological ly stained cells
and E shows the flourescent detection of eGFP); and (F & G) shows the ability of peptide 5 to
internalize eGFP in human dendritic cells (F is a photomicrograph of the histological ly stained
cells and G shows the flourescent detection of eGFP).

6 Figure 10 A-H shows the circular dichroism plot for the peptides 1-6 (SEQ ID
Nos:1-6 respectively) (Figures 10 A-F respectively), antennapedia peptide (SEQ ID NO:19)
(Figure 10 G) and a random peptide (SEQ ID NO:20) (Figure 10 H) at different wavelengths.

Figure 11 A & B: (A) Overlay of the CD spectra of peptide 4 (solid line, SED ID
NO:4), peptide 5 (dashed line, X, SEQ ID NO:5), TAT-PTD (solid line, ◆, SEQ ID NO:21), and
antennapedia peptide (solid line, ●, SEQ ID NO:19). (B) Overlay of the CD spectra of peptide
1 (solid line, ○, SEQ ID NO:1), peptide 2 (dashed line, ▲, SEQ ID NO:2), peptide 3 (dashed
line, ▼, SEQ ID NO:3), peptide 6 (solid line, □, SEQ ID NO:6).

Figure 12 shows the ability of various peptides (peptides 4, 5, random peptide,
antennapedia peptide, and TAT-PTD SEQ ID NO:4, SED ID NO:5, SEQ ID NO:20, SEQ ID
16 NO:19 and SED ID NO:21 respectively) to compete for binding for peptide 3, 4, 5, TAT-PTD,
antennapedia peptide, and a random peptide (SED ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ
ID NO:21, SEQ ID NO:19 and SEQ ID NO:20 respectively) in Hig-82 cells.

Figure 13 A-D: (A) fluorescence microscopy showing the ability of peptide 5
(SEQ ID NO:5) to internalize a streptavidin-488 flourescent marker and a Cy3 flourescent
21 marker into HIG-82 cells; (B) confocal microscopy showing the ability of peptide 5 to internalize

1 a streptavidin-488 fluorescent marker and a Cy3 fluorescent marker into HIG-82 cells; (C) shows the ability of peptide 5 linked to Cy3 to be internalized into HIG-82 cells; and (D) shows the ability of peptide 5 linked to M13 phase labeled with Cy3 to be internalized into HIG-82 cells.

Figure 14: shows the ability of the death peptide (SEQ ID NO:24), an antimicrobial apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) and peptide 5 at various concentrations to impair cell viability in HIG 82 Cells.

Figure 15: is a graph showing the ability of the death peptide (SEQ ID NO:24), an antimicrobial apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) and peptide 5 at various concentrations to impair cell viability in HIG 82 Cells as measured by OD₅₇₀ using an MTT assay.

Figure 16 (A) is a graph showing the ability of the death peptide (SEQ ID NO:24) (DP1; ●), antimicrobial peptide (SEQ ID NO:23) (KLA; ■) and peptide 5 (SEQ ID NO:5) (TBS; ▲) to inhibit the growth of MCA205 tumors, (B) shows representative surface morphology of mice with fibrosarcomas when treated with the death peptide (SEQ ID NO:24; DP1) and the antimicrobial peptide (SEQ ID NO:23; KLA) respectively, (C) is hematoxylin and eosin (H&E) staining (left) and TUNEL (right) showing that the death peptide (SEQ ID NO:24; DP1) but not the antimicrobial peptide alone (SEQ ID NO:23; KLA) mediates apoptosis of MCA205 tumors *in vivo*, and (D) is a scatter plot showing individual tumor sizes.

Figure 17 shows the ability of peptide 5 to facilitate the uptake of β -gal into CD34+/LIN- stem cells.

1 Figure 18 shows TUNEL hematoxylin and eosin staining of tissue from arthritic rabbit knee joints indicating that the death peptide (SEQ ID NO:24; DP1) mediates apoptosis in hyperplastic synovium whereas the antimicrobial peptide alone (SEQ ID NO:23; KLA) does not.

Figure 19 is a bar graph showing that the death peptide (SEQ ID NO:24; DP1) causes great reduction of white blood cells in lavage fluid of IL-1 inflamed rabbit joints.

6 Figure 20 is a bar graph showing that internalization of p53 into cells via the peptides of the present invention induces p21 promoter driven luciferase expression in a rabbit synovial cell line (Hig-82 cells).

Figure 21 shows fluorescence microscopy of HIG-82 cells treated with glutathione-pep5 (SEQ ID NO:5) linked to GST-eGFP (Panel A) or GST-eGFP alone (Panel B).

Figure 22 (A) shows a cross section of cervical mucosa treated with pep5-eGFP, (B) shows an optical orthogonal section through an explant of cervical mucosa, (C) shows a 3D reconstruction of cervical mucosa cells treated with pep5-eGFP viewed from within the tissue, and (D-G) shows flow cytometry analysis of a single cell suspension of cervical mucosa cells transduced with pep5-eGFP.

16 Figure 23 is a flow cytometry analysis of GM-CSF + IL4 propagated bone marrow-derived murine dendritic cells transduced with UBI-3Epi-eGFP recombinant protein indicating that the transduction of dendritic cells with an internalizing peptide/antigen complex promotes processing and subsequent presentation of dominant epitopes. (A) represents overnight culture of transduced dendritic cells with or without the presence of the proteasome inhibitor
21 MG132 and (B) represents overnight culture of dendritic cells transduced with UBI-3Epi-eGFP

1 and stained with D16.25 antibody that recognizes the OVA epitope within MHC H2-Kb molecule.

Figure 24 is a diagram depicting a fusion protein (UBI-3Epi-eGFP) comprising the UBI peptide (SEQ ID NO:73), Gp100₂₀₉₋₂₁₇ HLA-A2-restricted epitope, HIV p17₂₃₋₂₁ HLA-A2-restricted epitope, chicken ovalbumin (OVA) epitope and green fluorescent protein (eGFP).

6 DETAILED DESCRIPTION OF THE INVENTION

The peptides of the present invention facilitate the delivery, internalization and also, where desired, the cytoplasmic and/or nuclear transport of cargo into a wide variety of cell types. The delivery of cargo to a target cell is useful for various applications in gene therapy, oncology, developmental biology, the treatment of disease, immunogens, vaccines (*i.e.* eliciting an immune response) as well as for the general study of the mode of operation and the function of proteins, nucleic acids and small molecules in a model system. For example, a small molecule drug may be delivered to a cell via the peptides of the present invention, either *in vitro* or *in vivo* to study the effect of the drug on the cell (*e.g.* to see whether the drug induces apoptosis). Such delivery of small molecule drugs are useful for treating a wide variety of diseases, including arthritis and cancer. Additionally, a macromolecule or macromolecule complex, such as a protein, DNA, RNA, antisense RNA, virus, viral or non-viral vector etc., may be delivered to a cell via the peptides of the present invention, either *in vitro* or *in vivo* for the purpose of studying the effects of said macromolecule or macromolecular complex on the cell or to treat or otherwise affect a disease in a recipient requiring said macromolecule or macromolecular complex. For example, a macromolecule representing an apoptotic protein (*e.g.*

1 the apoptotic protein itself or a DNA encoding the apoptotic protein or a peptide with apoptotic
properties) may be delivered to synovial cells in arthritic joints or tumor cells to induce apoptosis
therein.

Model systems may include *in vitro* systems such as eukaryotic and prokaryotic
cell cultures, which can allow for the identification of the various components involved in a
6 particular biological pathway, the understanding of how a particular gene may be expressed or
how expression of a particular gene may be amplified and/or made persistent, the determination
of the function of a protein and how it may be inhibited, the determination of the function,
activity and mode of action of certain small molecules, as well as the feasibility of transfer into a
cell of particular cargo.

Model systems may also include animal model systems, which may aid in the
development of drugs for particular diseases, the determination of the efficacy of the up or down-
regulation of particular gene products *in vivo* and the resultant advantages or disadvantages of
such regulation and the determination of the efficacy of the delivery of proteins *in vivo* and
whether such delivery is efficient and effective for gene therapy or as a vaccine, etc. Such
16 information may give insight in the application of such methods in oncology, developmental
biology, gene therapy and vaccine development and may lead to new developments and a greater
understanding of disease and the treatment of disease, such as, but not limited to, the treatment of
arthritis and cancer.

The peptides of the present invention are useful, *inter alia*, for (1) facilitating the
21 uptake of cargo in a target cell; (2) inducing apoptosis in cells (*e.g.*, arthritic cells, tumor cells,

1 etc); (3) expanding a population of stem cells; (4) expanding a population of differentiated cells;
(5) stimulating the differentiation of a population of stem cells; (6) facilitating the integration of
AAV DNA into the genome of a cell; (7) facilitating the uptake into a cell, secretion from said
cell and subsequent reuptake into a neighboring cell of a protein; (8) facilitating the growth of
defective viruses in culture; (9) stimulating the immune response in a subject; (10) facilitating
6 uptake of any GST fusion protein, (11) eliciting an immune response in a subject; and (12)
facilitating the delivery of immunogens (*e.g.* vaccines), whether protein based, DNA based,
vector based or viral based.

In one embodiment, the present invention includes a complex comprising (a) an
internalizing peptide and (b) cargo. As used herein, a complex can be defined as two or more
molecules linked together by any physical means. The complex may be tightly or weakly linked
together in a highly specific or totally non-specific way. The internalizing peptides of the present
invention when linked to cargo facilitate the cellular uptake of cargo. As used herein, the term
“link” refers to any covalent cross-linkage or non-covalent linkage (*e.g.* a fusion protein
comprising the peptide and another protein) wherein said linkage is between the peptide of the
16 present invention and a cargo.

As used herein, “internalizing peptide” is a peptide that has been selected for its
ability to locate and enter a wide variety of cell types. Additionally, the internalizing peptides of
the invention may translocate into the nucleus of the cell. Furthermore the internalizing peptides
of the invention are capable of translocating and delivering cargo into a cell when linked to said
21 cargo. The peptides of the present invention are positively charged and amphipathic and may

1 interact with negative charges on the surface of the cellular bilayer membrane.

The internalizing peptides of the present invention may be complexed with cargo.

2 The term "cargo", as used herein, refers to any small molecule, macromolecule, or
3 macromolecular complex which may be useful to transfer to a cell. Cargo includes, but is not
4 limited to, small molecules, polynucleotides, DNA, oligonucleotide decoys, antisense RNA,
5 polypeptides, proteins, viruses, modified viruses, viral and non-viral vectors and plasmids. Small
6 molecules may be therapeutically useful and may include drugs or other agents which act to
7 ensure proper functioning of a cell or molecules which may induce apoptosis or cell lysis, where
8 death of a cell, such as a cancerous cell, is desired. Nucleic acids may code for, *inter alia*, a
9 protein, RNA, ribozyme, or antisense RNA. The protein, RNA or ribozyme encoded by the
10 nucleic acid may be under-represented, defunct or non-existent in the cell and the antisense RNA
11 encoded by the nucleic acid may allow for the elimination of an undesired function of a
12 molecule. Decoy oligonucleotides may contain specific binding sites for transcription factors
13 and may block the function of the transcription factors *in vitro* and *in vivo*. Where the cargo is a
14 polypeptide, the polypeptide may be a peptide or protein which, when delivered to the cell,
15 provides a desired function to the cell or induces a particular phenotypic alteration or the protein
16 or peptide may be an antigen capable of eliciting an immune response in the cell.

Amino acid residues in peptides are herein abbreviated as follows: Phenylalanine
is Phe or F; Leucine is Leu or L; Isoleucine is Ile or I; Methionine is Met or M; Valine is Val or
V; Serine is Ser or S; Proline is Pro or P; Threonine is Thr or T; Alanine is Ala or A; Tyrosine is
21 Tyr or Y; Histidine is His or H; Glutamine is Gln or Q; Asparagine is Asn or N; Lysine is Lys or

1 K; Aspartic Acid is Asp or D; Glutamic Acid is Glu or E; Cysteine is Cys or C; Tryptophan is Trp or W; Arginine is Arg or R; and Glycine is Gly or G.

6 The internalizing peptides of the present invention have been selected for their ability to be internalized into a wide variety of target cells. The internalizing peptides of the present invention obtained by M13 phage library screening with HIIG-82 cells are listed below in Table 1. The internalizing peptides of the present invention obtained by M13 phage library screening with human primary T cells are listed below in Table 2. The internalizing peptides of the present invention obtained by M13 phage library screening with Calu 3 cells (human epithelial cell line) are listed below in Table 3. The internalizing peptides of the present invention obtained by M13 phage library screening with surgically resected cervical mucosa tissue from human patients are listed below in Table 4. In addition, the internalizing peptides of the present invention may be identified by fusion of an internalizing peptide of the present invention with another peptide with a desired function such as, for example, but not by way of limitation, fusion of an internalizing peptide to a previously identified ubiquitin targeting peptide which may have the amino acid sequence GVVGKLGQRRTKKQRRQKK (the "UBI" peptide) as set forth by SEQ ID NO:73 or fusion of an internalizing peptide with an endoplasmaticum reticulum (ER) localization signal such as adenovirus E19 sequence which may have the amino acid sequence GRRTKKQRRQKKPPRYMILGLLALAAVCSAA as set forth by SEQ ID NO:74. In addition, the internalizing peptide of the present invention may have the amino acid sequence GRRTKKQRRQKKPP (SEQ ID NO:75).

21 Any technique known to those in the art may be employed to identify peptides

with high efficiency of internalization for a target cell. In one preferred embodiment of the invention, phage biopanning was employed to select for peptides (Table 1, 2, 3 and 4) which are internalized into such cell lines as Hig-82 cells, human synovial cells, rabbit synovial cells, human primary airway HBE144 cells, primary human islet cells, murine myoblast C2C12 cells, dog kidney epithelial MDCK cells, murine tumor MCA 205 cells, murine tumor MC38 cells (all shown in Figure 6) and rabbit synovial lining (Figure 4). In addition, the internalizing peptides are internalized into mucosa, such as cervical mucosa (*see* Figure 22, and Example 2 and 9 below). In another preferred embodiment, the internalizing peptides of the present invention can be fused to another peptide with a desired function (*e.g.* ubiquitin targeting or ER localization functions) such as the internalizing peptides set forth by SEQ ID NOs:73 and 74 which are also internalizing into a wide variety of cells including, but not limited to Hig-82 cells, human synovial cells, rabbit synovial cells, human primary airway HBE144 cells, primary human islet cells, murine myoblast C2C12 cells, dog kidney epithelial MDCK cells, murine tumor MCA 205 cells, murine tumor MC38 cells, rabbit synovial lining, and mucosa, such as cervical mucosa (*see* Figure 22, and Example 2 and 9 below)

TABLE 1

1)	peptide 1 (pep1)	KRIIQRILSRNS	(SEQ ID NO:1)
2)	peptide 2 (pep2)	KRIHPRLTRSIR	(SEQ ID NO:2)
3)	peptide 3 (pep3)	PPRLRKRRQLNM	(SEQ ID NO:3)
4)	peptide 4 (pep4)	PIRRRKKLRRLLK	(SEQ ID NO:4)
5)	peptide 5 (pep5)	RRQRRTSKLMKR	(SEQ ID NO:5)

1	6)	peptide 6 (pep6)	MHKRPTTPSRKM	(SEQ ID NO:6)
	7)	peptide 7 (pep7)	RQRSRRRPLNIR	(SEQ ID NO:7)
	8)	peptide 8 (pep8)	RIRMIQNLIKKT	(SEQ ID NO:8)
	9)	peptide 9 (pep9)	SRRKRQRSNMRI	(SEQ ID NO:9)
	10)	peptide 10 (pep10)	QRIRKSKISRTL	(SEQ ID NO:10)
6	11)	peptide 11 (pep11)	PSKRL LHNNLRR	(SEQ ID NO:11)
	12)	peptide 12 (pep12)	HRHIRRQSLIML	(SEQ ID NO:12)
	13)	peptide 13 (pep13)	PQNRLQIRRH SK	(SEQ ID NO:13)
	14)	peptide 14 (pep14)	PPHNRIQRRLNM	(SEQ ID NO:14)
	15)	peptide 15 (pep15)	SMLKRNHSTSNR	(SEQ ID NO:15)
	16)	peptide 16 (pep16)	GSRHPSLIIPRQ	(SEQ ID NO:16)
	17)	peptide 17 (pep17)	SPMQKTMNLPPM	(SEQ ID NO:17)
	18)	peptide 18 (pep18)	NKRILIRIMTRP	(SEQ ID NO:18)
	19)	peptide 19 (pep19)	HGWZIHGL LHRA	(SEQ ID NO:25)
	20)	peptide 20 (pep20)	AVPAKKRZKSV	(SEQ ID NO:26)
16	21)	peptide 21 (pep21)	PNTRVRPDVSF	(SEQ ID NO:27)
	22)	peptide 22 (pep22)	LTRNYEAWVPTP	(SEQ ID NO:28)
	23)	peptide 23 (pep23)	SAETVESCLAKSH	(SEQ ID NO:29)
	24)	peptide 24 (pep24)	YSHIATLPFTPT	(SEQ ID NO:30)
	25)	peptide 25 (pep25)	SYIQRTPTSTLP	(SEQ ID NO:31)
21	26)	peptide 26 (pep26)	AVPAENALNNPF	(SEQ ID NO:32)

1	27)	peptide 27 (pep27)	SFHQFARATLAS	(SEQ ID NO:33)
	28)	peptide 28 (pep28)	QSPTDFTFPNPL	(SEQ ID NO:34)
	29)	peptide 29 (pep29)	HFAAWGGWSLVH	(SEQ ID NO:35)
	30)	peptide 30 (pep30)	HIQLSPFSQSWR	(SEQ ID NO:36)
	31)	peptide 31 (pep31)	LTMPSDLQPVLW	(SEQ ID NO:37)

TABLE 2

	1)	peptide 32 (pep32)	FQPYDHPAEVSY	(SEQ ID NO:38)
	2)	peptide 33 (pep33)	FDPFFWKYSPRD	(SEQ ID NO:39)
	3)	peptide 34 (pep34)	FAPWDTASFMLG	(SEQ ID NO:40)
	4)	peptide 35 (pep35)	FTYKNFFWLPEL	(SEQ ID NO:41)
	5)	peptide 36 (pep36)	SATGAPWKMWVR	(SEQ ID NO:42)
	6)	peptide 37 (pep37)	SLGWMLPFSPPF	(SEQ ID NO:43)
	7)	peptide 38 (pep38)	SHAFTWPTYLQL	(SEQ ID NO:44)
	8)	peptide 39 (pep39)	SHNWLPLWPLRP	(SEQ ID NO:45)
	9)	peptide 40 (pep40)	SWLPYPWHVPSS	(SEQ ID NO:46)
16	10)	peptide 41 (pep41)	SWWTPWHVHSES	(SEQ ID NO:47)
	11)	peptide 42 (pep42)	SWAQHLSLPPVL	(SEQ ID NO:48)
	12)	peptide 43 (pep43)	SSSIFPPWLSFF	(SEQ ID NO:49)
	13)	peptide 44 (pep44)	LNVPWSWFLSQR	(SEQ ID NO:50)
	14)	peptide 45 (pep45)	LDITPFLSLTLP	(SEQ ID NO:51)
21	15)	peptide 46 (pep46)	LPHPVLHMGPLR	(SEQ ID NO:52)

1 16) peptide 47 (pep47) VSKQPYWMWNGN (SEQ ID NO:53)

TABLE 3

1) peptide 48 (pep48) NYTTYKSHFQDR (SEQ ID NO:54)

2) peptide 49 (pep49) AIPNNQLGFPEK (SEQ ID NO:55)

6 3) peptide 50 (pep50) NIENSTLATPLS (SEQ ID NO:56)

4) peptide 51 (pep51) YPYDANHTRSPT (SEQ ID NO:57)

5) peptide 52 (pep52) DPATNPGPHFPR (SEQ ID NO:58)

6) peptide 53 (pep53) TLPSPALLTVH (SEQ ID NO:59)

7) peptide 54 (pep54) HPGSPFPPEHRP (SEQ ID NO:60)

8) peptide 55 (pep55) TSHTDAPPARSP (SEQ ID NO:61)

9) peptide 56 (pep56) MTPSSLSTLPWP (SEQ ID NO:62)

10) peptide 57 (pep57) VLGQSGYLMPMR (SEQ ID NO:63)

TABLE 4

1) peptide 58 (pep58) QPIITSPYLP (SEQ ID NO:64)

16 2) peptide 59 (pep59) TPKTMTQTYDFS (SEQ ID NO:65)

3) peptide 60 (pep60) NSGTMQSASRAT (SEQ ID NO:66)

4) peptide 61 (pep61) QAASRVENYMR (SEQ ID NO:67)

5) peptide 62 (pep62) HQHKPPPLTNNW (SEQ ID NO:68)

6) peptide 63 (pep63) SNPWDSLLSVST (SED ID NO:69)

21 7) peptide 64 (pep64) KTIEAHPPYYAS (SEQ ID NO:70)

1 8) peptide 65 (pep65) EPDNWSLDFPRR (SEQ ID NO:71)

 9) peptide 66 (pep66) HQHKPPPLTNNW (SEQ ID NO:72)

 Of the peptides of Table 4, three have homology to known proteins. Pep63 (SEQ ID NO:69) is homologous to a bacterial protein methenyl tetrahydromethanopterin cyclohydrolase of xanthobacter autotrophicus (Genbank Accession Number AF139593). Pep65 (SEQ ID NO:71) is homologous to a yeast hypothetical protein in the MPP10-SAG1 intergenic region of Saccharomyces cerevisiae (Genbank Accession Number NP012536.1). Additionally, pep66 (SEQ ID NO:72) is homologous to herpesvirus 1 probably nuclear antigen protein (Genbank Accession Number P33485).

 Additionally, any nucleotide sequences which encode the peptides represented by SEQ ID NOs:1-18 and 25-72 of the present invention are also contemplated by the present invention.

 The internalizing peptides of the present invention are cationic (positively charged) as are TAT-PTD and antennapedia peptide (Ant-PTD). Table 5 below indicates the positive nature of pep 1-6 (SEQ ID NO:1-6), TAT-PTD (SEQ ID NO:21), Ant-PTD (SEQ ID NO:19) and a random control peptide (SEQ ID NO:20). Peptides 1-6 are all positively charged and enriched for lysine and arginine residues (Table 5 below).

TABLE 5

<u>Peptide</u>	<u>SEQ ID NO.</u>	<u>Length</u>	<u>MW</u>	<u>Lys + Arg/L</u>
Pep 1	SEQ ID NO:1	12	1482.81	0.333
Pep 2	SEQ ID NO:2	12	1531.88	0.417

1	Pep 3	SEQ ID NO:3	12	1563.94	0.417
	Pep 4	SEQ ID NO:4	12	1619.81	0.667
	Pep 5	SEQ ID NO:5	12	1614.98	0.583
	Pep 6	SEQ ID NO:6	12	1468.80	0.333
	Random	SEQ ID NO:20	12	1280.40	0.167
6	TAT-PTD	SEQ ID NO:21	11	1558.63	0.727
	Ant-PTD	SEQ ID NO:19	16	2245.78	0.438

Where desired, the internalizing peptides of the present invention may also facilitate the nuclear translocation of cargo.

The usefulness of the present invention may be demonstrated, for example, by incubating a complex comprising an internalizing peptide linked to cargo with target cells and measuring the efficiency of transfer of the peptide-cargo complex to the target cell. In one embodiment, the selected internalizing peptide was biotinylated and coupled to streptavidin-labeled β -galactosidase (the "cargo"). The ability of the internalizing peptide to internalize β -gal into a cell was established by adding X-gal to cells, which when in the presence of β -gal is cleaved and gives a blue color. Cells which stained blue indicated that β -gal had successfully been transferred to the cells via the peptides of the present invention (Fig. 1-6 and 12) (see Example 4 below). In addition, a polynucleotide encoding one of the peptides (peptide 5; SEQ ID NO:5) was subcloned with nucleic acid encoding eGFP (green fluorescent protein) to produce a peptide-eGFP fusion, expressed and purified from bacteria. Peptide 5, when fused to eGFP, facilitated the internalization of eGFP into cells (see Figure 9), which was directly monitored by

1 fluorescence microscopy.

2 In a preferred embodiment of the invention, the internalizing peptides which allow
3 for the co-entry of peptide-linked cargo, and the translocation of the cargo to the nuclei are pep 2
4 (SEQ ID NO:2), pep 3 (SEQ ID NO:3), pep 4 (SEQ ID NO:4), pep5 (SEQ ID NO:5) shown in
5 Table 1 above, and UBI (SEQ ID NO:73). The internalizing peptides of the present invention
6 may be linked to cargo by any method known to those in the art, such as, but not limited to
chemical cross-linking, avidin bridge, glutathione-S-transferase bridge, peptide-cargo fusion
protein, etc. The peptides of the present invention may also be synthesized as a fusion with a
peptide nucleic acid (PNA) which is a DNA mimic capable of forming double and triple helices
with DNA (see Knudsen and Nielsen, 1997, *Anticancer Drugs* 8:113-118). This peptide-PNA
fusion can form a stable DNA or RNA/PNA duplex (Branden et al., 1999, *Nat. Biotechnol.*
17:784-787) which may enter cells via the peptides of the present invention, thereby delivering
DNA or RNA to a target cell.

16 Additionally, the ability of the internalizing peptide to carry the cargo into the cell
may be measured by the presence of functional cargo in the cell (e.g. the presence of β -gal may
be demonstrated by the ability of the cell to cleave X-gal and give a blue color; the presence of
cystic fibrosis transmembrane regulator (CFTR) protein may be demonstrated by the presence of
a functional chloride ion channel in a cell originally lacking CFTR, and the presence of an
apoptotic factor may be shown by the apoptosis of cells after the administration of a peptide-
apoptosis factor construct of the present invention). The cargo (e.g. polypeptide, polynucleotide,
21 small molecule, virus, plasmid) may be labeled by a method known in the art (e.g. radiolabeling

1 or fluorescent labeling) and the presence of the label would establish the efficient delivery of the
cargo into the target cell by the internalizing peptide. In addition, the presence of an immunogen
in the cell of a subject may be measured by the ability to elicit an immune response in a subject.

To establish nuclear translocation of the internalizing peptides themselves and the
ability of the internalizing peptide to transfer a small molecule linked thereto to a cell, the
6 peptides were labeled with streptavidin-Cy3, a fluorescent marker (see Example 4 below). Using
confocal microscopy, the ability of the peptide to translocate to the nucleus is determined. Other
methods known in the art of establishing the presence of a peptide in the cytoplasm or nucleus of
a cell are also contemplated by the present invention (*e.g.* labeling of the peptide with a
radioisotope, a fluorescent marker or a dye).

The internalizing peptides of the present invention facilitate uptake and delivery
into a wide variety of cell types (see Figures 1-4 and 6) including cells which are refractory to
virus infection, such as primary human airway epithelial cells (Figure 6), as well as other types of
primary and established cell lines, such as Hig-82 cells (a rabbit synovial cell line established by
Christopher Evans, University of Pittsburgh, ATCC Deposit No. CRL-1832), rabbit synovial
16 cells, human synovial cells, primary human islet cells, murine myoblast cells, dog kidney
epithelial cells, murine fibroblast cells, and murine tumor cells (Figures 1-4 and 6) (see Examples
4 and 5 below), cells of different germinal layers, as well as mucosa, such as cervical mucosa
(Figure 22).

The peptides of the present invention are also useful for delivery of cargo into
21 cells *in vivo* and can facilitate *in situ* or localized delivery of cargo *in vivo* (see figure 4 and

1 Example 3 below). In one embodiment, a biotinylated peptide-streptavidin- β -gal complex was
 injected into synovial lining (knee joint) of rabbits, which was then harvested. The harvested
 synovial lining was then incubated with X-gal to show that the peptide facilitated the uptake of
 β -gal by synovial lining cells *in vivo*. Ghivizzani et al. (*J. Immunol.* 159:3604 (1997)) have
 described using the synovial lining of rabbits as a model system for studying arthritis (*see also*
 6 Nita et al., *Arthritis Rheum* 39:820 (1996); Ghivizzani et al., *Proc. Natl. Acad. Sci. USA* 95:4613
 (1998); and Ghivizzani et al., *Gene Ther.* 4:977 (1997)). Rheumatoid arthritis is correlated with
 an excessive proliferation of synovial cells and an apparent defect in synovial cell death that
 would ordinarily reduce the synovial cell number. Because the peptides of the present invention
 can facilitate the uptake of cargo into synovial lining cells *in vivo*, the peptides are useful in the
 alleviation of arthritis. One approach to alleviating rheumatoid arthritis in a subject is to induce
 synovial cell death. *See* Wakisaka et al., *Clin. Exp. Immunol.* 114:119-128 (1998); Sakai et al.,
Arthritis Rheum. 41:1251-1257 (1998).

The peptides of the present invention, as well as TAT-PTD, can induce apoptosis
 in rheumatoid arthritis synovial cells when linked to an apoptosis factor. For example, the
 16 peptides of the present invention, as well as TAT-PTD, when linked to an apoptosis factor (*e.g.*
 p53; caspase-3; an antimicrobial peptide such as KLAKLAK (SEQ ID NO:22) and
 KLAKLAKKLAKLAK (SEQ ID NO:23), which disrupts the mitochondrial membrane once
 inside a cell (*see* Ellerby et al., *Nat. Med.* 5:1032 (1999)); are useful for delivering the apoptosis
 factor, or a DNA encoding an apoptosis factor, to arthritic joints and inducing apoptosis therein
 21 (*see* Figure 18 and Example 7 below). In addition, the peptides of the present invention, as well

as TAT-PTD, are useful for delivering apoptosis factors to tumor cells and inducing apoptosis therein. The induction of apoptosis in tumor cells is useful for the destruction of the tumor cell and for increasing the efficacy of drugs designed to treat cancer which are ineffective in tumor cells resistant to apoptosis. See Brown and Wouters, *Cancer Res.* 59:1391-1399 (1999); Yamabe et al., *Gene Ther.* 6:1952-1959 (1999). When the antimicrobial peptide, KLAKLAKKLAKLAK (SEQ ID NO:23), is coupled to the peptides of the present invention or TAT-PTD (e.g. the "death peptide" = peptide 5, SEQ ID NO:5, a linker and KLAKLAKKLAKKLAK (SEQ ID NO:23) resulting in RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24)) and administered to HIG 82 cells, apoptosis was induced in the cells (see Figures 14 and 15 and Example 7 below). Furthermore, when the "death peptide" was intra-tumorally injected subcutaneously into day 7-14 MCA205 (a murine fibrosarcoma cell line) established tumors in mice (the following references describe using MCA205 cells to establish tumors in mice for a model system for studying cancer: Hiroishi et al., *Gene Ther.* 6:1988 (1999); Osaki et al., *Gene Ther.* 6:808 (1999); Nishioka et al., *Cancer Res.* 59:4035 (1999); Gambotto et al., *Cancer Gene Ther.* 6:45 (1999); Kim et al., *Cancer Immunol Immunother.* 47:257 (1999); Mangency and Heidmann, *Proc. Natl. Acad. Sci. USA* 95:14920 (1998); Noffz et al., *J. Immunol.* 160:345 (1998); Osaki et al., *J. Immunol.* 160:1742 (1998); Cayeux et al., *J. Immunol.* 158:2834 (1997); Ohno et al., *J. Immunol.* 156:3875 (1996)), there was shrinkage of the tumor with significant apoptosis/necrosis, especially in the middle of the tumor (see Figure 16A, 16B, 16C and Example 7 below).

As noted above, the internalizing peptides of the present invention are useful for

1 delivering and internalizing other apoptotic factors as well, including p53. When p53 was fused
to pep5 (SEQ ID NO:5), the p53 was effectively internalized into a rabbit synovial cell line (Fig-
82) and able to induce p21 promoter driven luciferase expression from a reporter plasmid therein
(see Figure 20 and Example 7 below). The pep5-p53 complex was similar in its ability to induce
reporter plasmid expression as a plasmid which expresses p53 and much more effective than an
6 adenovirus vector expressing p53 (see Figure 20 and Example 7 below). Due to its apoptotic
abilities, the internalizing peptide-p53 complex of the present invention is useful in the treatment
of cancer and arthritis and may be administered to a subject, for example, by either local or
systemic injection (such as intra-tumoral injection or intra-articular injection).

The "death peptide" is also useful for the induction of apoptosis in other cells,
including synovial lining cells. When the death peptide was injected in the arthritic rabbit knees,
it mediated apoptosis of the hyperplastic synovium (see Figure 18 and Example 7 below).
Internalization of apoptosis factors using the peptides of the present invention and TAT-PTD is
advantageous since cellular uptake of cargo mediated by the peptides of the present invention is
more efficient than viral vector mediated gene transfer or the commercially available
16 antennapedia peptide (Example 3 and Figure 4A).

Rheumatoid arthritis (RA) is a chronic inflammatory disease which is
characterized by hyperplasia of the synovial lining of cells, angiogenesis, and infiltration of
mononuclear cells resulting in pannus formation, cartilage erosion and ultimately joint
destruction. Most of articular cartilage consists of collagens and proteoglycans whose
21 degradation is initiated extra- or peri- cellularly by proteinases produced locally by cells in a

1 around the joint. *See Evans, Agents Actions Suppl.* 32:135-152 (1991). Proteinases, and
particularly serine proteinases and neutral metalloproteinases, are involved in the degradation of
articular cartilage. Mesenchymal cells of the joint and white blood cells which colonize the joint
during the inflammatory response synthesize various proteinases which degrade articular
cartilage. Therefore, reduction of white blood cells at the site of inflammation in arthritic joints
6 is an approach to anti-erosive therapy in arthritis. The internalizing peptides of the present
invention are useful in delivering apoptotic factors to cells in arthritic joints, including white
blood cells in lavage fluid of inflamed arthritic joints. Figure 19 shows that injection of the death
peptide (SEQ ID NO:24; DP1) into inflamed rabbit joints causes a great reduction of the number
of white blood cells in the lavage fluid of IL-1 inflamed rabbit joints (*see also* Example 7 below).
White blood cell reduction is useful to reduce swelling, synovial proliferation and cartilage
degradation in arthritic joints.

Delivery of apoptosis factors via the peptides of the present invention is rapid and
potent. For example, low concentrations of the death peptide (SEQ ID NO:24) are required to
mediate cell death. In one embodiment of the invention, cell death may be mediated by the
16 internalizing peptides of the present invention (SEQ ID NOs:1-18 and 25-72) linked to cargo
comprising an apoptosis factor wherein the concentration administered to cells is between 1 μ M
and 1mM. In a preferred embodiment of the present invention, the concentration of the peptide +
cargo administered to cells is between 10 μ M and 100 μ M.

In another aspect of the invention, the immune response against tumors may be
21 augmented by co-administration of the internalizing peptides of the present invention linked to a

1 cargo (e.g. apoptosis factor) with cytokines and other activating molecules (e.g. Flt-3). The
cytokines and other activating molecules may be administered to cells via the peptides of the
present invention or by any other conventional means of administration known to those of skill in
the art.

6 Additional potential applications for the peptides of the present invention when
linked to cargo comprising an apoptosis factor may include the treatment of accessible head and
neck tumors, papillomas and other solid tumors, or as an adjuvant therapy in conjunction with
radiotherapy, standard chemotherapy or surgical debulking to extend excision margins.

The peptides of the present invention are also useful for developing improved
immunogens. For example, the peptides of the present invention may facilitate delivery of, *inter*
alia, proteins, polypeptides, DNA, RNA, vectors, and viruses to target cells in a subject which
may be useful as immunogens. The peptide/cargo complexes of the present invention are
capable of eliciting an immune response when administered to a target cell of a subject. In one
embodiment of the invention, the immunogens are vaccines.

16 While intense efforts have been made in engineering vaccines for HIV in the past
decade, an effective vaccine has yet to be developed. The peptides of the present invention may
be useful for the development of an effective vaccine for HIV. It is one object of the present
invention to provide a vaccine for HIV which is effective at mucosal portals of entry and is
capable of eliciting an immune response when delivered to target cells.

21 The existence of a "common mucosal immune compartment" distinct from
systemic immunity is well documented. See Miller et al., *Lab. Invest.* 68:129-145 (1993) and

1 James, *New Generation Vaccines*, edited by Levine M., Woodrow GC, Kaper JB and Cobon BS.
Marcel Dekker, Inc., New York pages 151-171 (1995). The mucosal immune system is
compartmentalized into "inductive" sites of mucosally associated lymphoid tissue (*e.g.* Peyer's
Patches) where antigen priming occurs and "effector" sites (*e.g.* lamina propria and epithelium of
mucosal tissue) where primed mature effector cells protect against invasion of foreign agents.

6 *See* Haneberg et al., *Adv. Exp. Med. Biol.* 371A:107-109 (1995). In the intestine, antigen-
stimulated induction of naive T and B lymphocytes in the Peyer's patches is followed by
trafficking of these cells through adjacent draining lymph nodes (*e.g.* mesenteric lymph nodes).
Fully mature effectors finally traffic to remote lamina propria of mucosal tissues via circulation
through the thoracic duct and blood.

The present invention provides a method of eliciting an immune response and for
immunogens (such as HIV vaccines). Since the primary mode of transmission of HIV is via
sexual intercourse, the immunogens of the present invention can induce specific mucosal
immune responses.

The immunogens of the present invention preferably comprise an internalizing
peptide portion linked to cargo (*e.g.* antigen). The immunogens of the present invention can
present antigen directly to any target cell (*e.g.* mucosal inductive sites).

The immunogens of the present invention can efficiently induce an immune
response, *e.g.* T helper cell type 1 (TH1) immune responses. T-cells recognize antigens only if
they are presented in the form of short, linear peptides (epitopes) in the cleft of major
histocompatibility complex (MHC) molecules on the cell surface. Therefore, if antigens are to

1 be recognized, they must first be processed into short, linear peptides. Most proteins in the
 cytosol are cleaved by proteases within proteosome complexes into short peptides and carried by
 transporter proteins into the endoplasmic reticulum (ER). In the ER, the peptides are bound to
 MHC molecules that are synthesized in the ER. The MHC molecules are then transported to the
 cell surface where the peptide bound to the MHC molecule is recognized by T-cell receptors (*see*
 6 Figure 23).

Figure 23 demonstrates that transduction of a target cell, *e.g.*, dendritic cells, with
 the peptide/cargo complex of the present invention can promote processing and subsequent
 presentation of epitopes on the surface of the target cell. To demonstrate that the peptide/cargo
 complex of the present invention can promote processing and subsequent presentation of
 epitopes on the surface of a target cell, UBI (SEQ ID NO:73) was fused to a peptide comprising
 Gp100₂₀₉₋₂₁₇ HLA-A2-restricted epitope, HIV p17₂₃₋₂₁ HLA-A2-restricted epitope and chicken
 ovalbumin (OVA) epitope (termed 3-Epi) which was in turn fused to green fluorescent protein
 (eGFP), to make UBI-3Epi-eGFP (*see* Figure 24). As seen in Figure 23, the UBI-3Epi-eGFP
 fusion was efficiently degraded by the proteasome complex in dendritic cells since the
 16 degradation is blocked by the proteasome inhibitor MG132 (*see* Figure 23A). Furthermore, class
 I presentation and T cell specific recognition of epitope on the surface of mouse dendritic cells is
 shown in Figure 23B using D16.25 antibody staining which recognizes the OVA epitope of UBI-
 3Epi-eGFP.

CD8⁺ cytotoxic T-lymphocytes generally recognize 8-11mer peptides on MHC
 21 class I molecules, whereas CD4⁺ T-helper cells generally recognize 15-25mer peptides on MHC

1 class II molecules. The presentation of the short, linear peptides of the antigen on the cell surface
by MHC molecules allows for the initial steps required for T-cell activation. Once T-cell
activation is achieved, a potent immune response may be elicited.

6 The present invention provides for immunogens which may comprise an
internalizing peptide of the present invention linked to cargo such as a protein representing an
antigen or a DNA or RNA encoding for an antigen. The present invention also provides for a
method for eliciting an immune response to a target cell, said method comprising delivering an
immunogen comprising an internalizing peptide and a cargo (preferably an antigen) to a target
cell. Once the immunogen is delivered to the target cell, the cargo may be processed (*e.g.* where
the cargo is an antigen, the cargo is proteolyzed into short, linear peptides, or, where the cargo is
an RNA or DNA encoding an antigen, the cargo is expressed and then proteolyzed into short,
linear peptides) and presented to the cell surface thereby eliciting an immune response (*see*
Figure 23). The target cell may be a mucosal cell such as a cervical mucosal cell (*see* Figure 22)

16 In a preferred embodiment of the invention, the immunogen comprises pep5 (SEQ
ID NO:5) linked to cargo, such as an antigen. In another preferred embodiment of the invention,
the immunogen comprises the UBI peptide (SEQ ID NO:73) linked to cargo, such as an antigen.
In a further preferred embodiment of the invention, the immunogen comprises SEQ ID NO:74
linked to cargo, such as an antigen.

21 The cargo portion of the immunogens of the present invention may be an antigen
capable of eliciting an immune response to HIV exposure, such as, *inter alia*, an HIV envelope
protein, HIV Gag, HIV Pol, HIV Env, HIV Tat, HIV Nef, HIV Vpr, HIV Vpv and HIV Rev. Or

1 the cargo portion of the immunogens of the present invention may be any antigen capable of
eliciting a desired immune response.

The immunogens of the present invention and the methods of the present
invention for eliciting an immune response in a subject can also be accomplished by *ex vivo*
transduction of target cells followed by the presentation of the transduced cells to a subject by,
6 for example, intra-muscular or intra-dermal injection or any other technique known to the skilled
artisan.

The method of the present invention for eliciting an immune response in a subject
comprises administering to a target cell of said subject (whether *in vitro*, *in vivo*, or *ex vivo*) a
peptide/cargo complex of the present invention wherein said peptide is selected from SEQ ID
NOs:1-74 and the cargo is an antigen.

The peptide-cargo complexes of the present invention may be administered to a
wide variety of cell types *in vivo*, *in vitro*, and *ex vivo* including, *inter alia*, epithelial cells, tumor
cells, hepatocytes, endothelial cells, neurons, muscle, T-cells, dendritic cells, β cells, primary
cells, differentiated cells, stem cells, antigen presenting cells, mucosa, etc by methods known to
16 those skilled in the art.

When administered to stem cells (*e.g.* hematopoietic, muscle, brain, etc.), the
peptide-cargo complexes of the present invention can induce differentiation of the stem cells.
The peptide cargo complex comprises factors which can stimulate differentiation of stem cells,
such as the transcription factor MyoD. Stem cells isolated from bone marrow have been shown to
21 differentiate into a wide variety of tissues, including cartilage and bone, and may be useful

1 therapeutically. See Pittenger et al., *Science* 284:143 (1999).

2 In addition, the peptide-cargo complex may be used to expand a stem cell
3 population. The internalizing peptides of the present invention can deliver proteins to CD34+
4 hematopoietic progenitor stem cells (see Figure 17 and Example 4). The delivery of
5 immortalizing proteins, such as SV40 T-antigen, HPV E6, HPV E7 and telomerase, can facilitate
6 the transient expansion of stem cell populations. Since the delivery of the immortalizing proteins
7 using the peptides of the present invention is transient and reversible (e.g. delivery of the
8 immortalizing protein which will be degraded subsequently in the cell), such delivery offers an
9 advantage in that the stem cell status may be maintained (i.e. the cells may be transiently
10 immortalized) while increasing the number of cell doublings that may be achieved. Stable
11 delivery of immortalizing factors may also be achieved by the delivery of cargo encoding the
12 immortalizing factor, e.g. a viral vector, plasmid, DNA. This approach can be used to expand a
13 wide variety of stem cells in culture for transplant applications since the peptides of the present
14 invention can facilitate the uptake and delivery of cargo linked thereto to a variety of cells (see
15 Example 3 and Figure 6).

16 Similarly, the peptides of the present invention may be used for expanding
17 differentiated cells (e.g. β cells in pancreatic islets, neurons, chondrocytes, etc) which also have a
18 finite number of cell doublings in culture. The peptides of the present invention enter and
19 facilitate the internalization of cargo in differentiated cells, such as islet β cells, (see Example 3,
20 and Figure 6) without affecting the ability of the islet cells to respond to signals which are
21 indicative of differentiated function, such as glucose. The proliferation of differentiated cells

1 may be induced by delivering immortalizing factors (*e.g.* SV40 T-antigen, HPV E6, HPV E7 and telomerase), and particularly SV40 T-antigen, complexed to the peptides of the present invention. The delivery may be transient (delivery of the protein) or may be stable (delivery of a DNA, viral vector, or plasmid encoding the immortalization factor).

6 It is also an object of the present invention to provide a construct comprising a peptide of the present invention linked to an antigen which can be taken up efficiently by a number of antigen presenting cells (*e.g.* dendritic cells) both *in vivo* and *in vitro* and stimulate an immune response. The peptides may be linked to, *inter alia*, viral antigens (*e.g.*, HIV antigens such as Gag, Pol, Env; HPV-E6; HPV-E7; EBV-LMP1; EBV-LMP2; EBNA1; EBNA3A; EBNA3C; etc), ovalbumin, differentiation antigens (*e.g.*, MART-1/Melan A, gp100, tyrosinase, TRP-1, TRP-2, etc.), tumor specific multilineage antigens (*e.g.*, MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, p15, etc), antigens expressed uniquely by an individual's tumor (*e.g.*, mutated gene products such as p53, CDK4, p16, p21, etc.). In one embodiment, the peptides of the present invention are linked to SIV antigens and are delivered *in vivo* to monkeys to determine the efficacy of said constructs in an *in vivo* system.

16 In another embodiment, the peptides of the present invention when complexed to the adeno-associated virus (AAV) Rep protein, can facilitate the integration of AAV DNA (and any DNA inserted into an AAV vector) into the genome of a target cell. The Rep protein of adeno-associated virus is able to facilitate integration of the AAV genome into a specific site on human chromosome 19. *See* Weitzman et al., *Proc. Natl. Acad. Sci. USA* 91:5808-5812 (1994).

21 However the Rep protein is toxic and is difficult to deliver to cells as a DNA encoding the

1 protein. In fact, to date it has not been feasible to generate a stable cell line constitutively
expressing Rep or an adenoviral helper vector that can transiently express Rep. The present
invention provides a complex comprising a peptide of the present invention (*e.g.* peptide 2, 3, 4
or 5) linked to the Rep protein. Such a complex facilitates the delivery of the Rep protein to a
target cell. The target cell can be infected with AAV (or transfected with AAV DNA) before or
6 after treatment with the peptide-Rep complex. The presence of the peptide-Rep complex and the
AAV DNA allows for the integration of the AAV DNA into the target cell genome.

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The present invention is further directed to promoting the growth of defective
viruses, such as HSV, in culture. The generation of defective viruses is useful for gene therapy
applications. Defective viruses do not replicate without the help of necessary replication proteins
which are not encoded by such defective viruses. One approach has been to construct cell lines
expressing the necessary viral replication proteins (*e.g.* ICP0, ICP4, ICP22 and ICP27), which
have been difficult to generate. In one embodiment of the present invention, replication
defective virus is grown in cells by infecting the cells with the defective virus and administering
one or more complexes comprising a peptide of the present invention linked to a protein
16 necessary for the replication of the defective virus.

GST fusion proteins are widely used in research to study various proteins due to
the ease of expressing and purifying such fusion proteins. The internalizing peptides of the
present invention are useful for a universal system for delivering any GST fusion protein to cells.
The GST fusion protein may be made by techniques known in the art, such as the method
21 described by Pharmacia (Piscataway, NJ). The peptides of the present invention, when linked to

1 glutathione, can facilitate the delivery of GST fusion proteins in a target cell. The glutathione-peptide constructs of the present invention can bind to any GST fusion protein and facilitate the internalization of the GST fusion protein into a cell (*see* Figure 21 and Example 8 below). The present invention is also directed to kits comprising the glutathione-peptide construct.

6 It is also an object of the present invention to provide an expression cassette comprising a nucleic acid encoding a fusion protein comprising a leader sequence, an internalizing peptide of the present invention, and a protein of interest, operably linked to expression control sequences. Such a fusion protein is capable of post-translational intercellular transport via the leader sequence or the internalizing peptides of the present invention. The leader sequence may be derived from secreted gene products such as interleukin-1 receptor antagonist (IL-1ra), Parathyroid hormone (PTH), or cathelin (*see* Huttner et al., *Ped. Res.* 45:785 (1999)). Since the leader sequence may be clipped or removed during translocation, the internalizing peptides of the present invention ensure that the fusion protein encoded by the expression cassette of the present invention may still be internalized into surrounding cells even after removal of the leader sequences, thereby improving the efficiency of intercellular transport.

16 The protein of interest may include, *inter alia*, apoptotic proteins, suicide proteins, therapeutic proteins, etc.. In addition, a herpes simplex virus protein, VP22, has been shown to be released from cells and taken up by neighboring cells. *See* Elliot & O'Hare, *Cell* 188:223-233 (1997); Elliot & O'Hare, *J. Virol.* 74:2131-2141 (2000); Derer et al., *J. Mol. Med.* 77:609-613 (1999).

Another embodiment of the present invention is directed to a fusion construct comprising the leader sequence of VP22, a peptide of the present invention (preferably peptide 2, 3, 4 or 5) and a

1 protein capable of achieving a desired effect in a cell (e.g. apoptotic protein, suicide protein,
therapeutic protein, etc).

2 The expression cassette of the present invention may further comprise expression
3 control sequences operably linked to the nucleic acid encoding the fusion protein and may be
4 contained within a transfer vector which may be administered to cells either *in vivo* or *in vitro*
5 and mediate expression therein. In addition to containing the DNA sequences encoding one or
6 more transgenes, the chimeric adenoviral vectors of the invention may contain any expression
control sequences such as a promoter or enhancer, a polyadenylation element, and any other
regulatory elements that may be used to modulate or increase expression, all of which are
operably linked in order to allow expression of the transgene. The use of any expression control
sequences, or regulatory elements, which facilitate expression of the transgene is within the
scope of the invention. Such sequences or elements may be capable of generating tissue-specific
expression or be susceptible to induction by exogenous agents or stimuli. For example, suitable
promoters include promoters such as from phosphoglycerate kinase (PGK) promoter or a
cytomegalovirus (CMV). In one embodiment, a vector containing the expression cassette
16 comprising DNA sequences encoding a fusion protein comprising a leader sequence, an
internalizing peptide and a protein of interest is administered to a cell wherein said expression
cassette is transcribed and translated and the resultant fusion protein is then secreted via the
leader sequences. After secretion from the cell in which it was expressed, the fusion protein
comprising an internalizing peptide, therapeutic protein or other protein of interest, and
21 optionally the leader sequence (which may alternatively be cleaved) may be internalized into

1 surrounding cells *in vivo* or *in vitro* via the internalizing peptides of the present invention.

Such an expression cassette is useful for sustained delivery of a peptide-cargo complex in cells. Any leader sequence capable of directing the secretion of a polypeptide linked thereto is contemplated by the present invention, including, but not limited to IL-1ra, PTH and related sequences. The expression cassette comprising DNA sequences encoding a fusion
6 protein comprising a leader sequence, an internalizing peptide and a protein of interest is useful to direct the delivery of the protein of interest to surrounding cells. The protein of interest may be an apoptotic protein, anti-apoptotic protein, cell cycle regulatory protein, transcription factor, suicide gene product, viral or tumor antigens, or cell proliferation factors (*e.g.* viral oncoproteins, telomerase, etc.).

The invention is also related to methods of identifying the internalizing peptides of the present invention. Peptides having the ability to be internalized into cells can be identified by random peptide libraries coupled with an affinity enrichment process. A phage display peptide library kit, such as that supplied by New England Biolabs, Inc. (Beverly, MA) may be employed in the present invention for the identification of peptides which are capable of being internalized into
16 cells and are also capable of facilitating the internalization of cargo into cells. A random peptide library may also be presented on a plasmid (as part of a fusion protein) or protein as a peptide-protein complex by techniques known in the art. Methods of identifying internalizing peptides can facilitate the isolation of peptides with superior internalizing capabilities and provide numerous peptides which can be selected for a reduced likelihood of eliciting an immune response when
21 administered to a subject and an increased half life *in vivo* and/or *in vitro*.

1 The method comprises (a) incubating a target cell with a peptide display library; (b) isolating internalized peptide presented by said peptide display library from the cytoplasm and nuclei of the cells and identifying said peptides; (c) linking said peptides to cargo; (d) incubating said peptide-cargo complex with a target cell; and (e) determining ability of said peptides to facilitate the uptake and, where desired, nuclear localization of said cargo into said target cell.

6 In a preferred embodiment, a random peptide library is presented on the surface of bacteriophage M13 as coat protein fusions creating a physical linkage between the displayed peptide and its encoding DNA sequence. *E.g.*, New England Biolabs, Inc. Ph.D.TM phage display peptide library kits (Beverly, MA). Such phage display peptide libraries allow for the selection of peptide ligands for a variety of targets through biopanning, including panning against intact cells. *See* Barry and Johnston, 1996, *Nature Medicine* 2:299-305; Szardenings et al., 1997, *J. Biol. Chem.* 272:27943-27948. Panning against intact cells may allow for the identification of peptides which facilitate the internalization of the phage on which they are displayed. *See* Vasily et al., 1999, *Biochimica et Biophysica Acta* 1448:450-462; and Vasily et al., 1999, *Biochimica et Biophysica Acta* 1448:463-472. Additionally, a T7 phage display library, which is able to express larger peptides fused to the carboxyl terminus of the T7 phage 10B fiber protein (as compared to a 12 amino acid peptide library which is expressed on the coat of the New England Biolabs M13 library) may also be employed for biopanning.

16 The phage display peptide library may be incubated with a target cell line (*e.g.* Hig-82 cells) to isolate phage which are internalized into the cells (see Examples 2 and 3 below). The cells are then harvested and lysed to isolate the internalized phage which express peptides which are

21

1 capable of facilitating their internalization. The cell lysate is collected for phage titering and
amplification in bacteria. The procedure is repeated with amplified phage a total of three times to
obtain phage preparations which are enriched for the peptides responsible for the internalization of
the phage. After three rounds of biopanning, titering and amplification, the phage are used to infect
bacterial lawns for the purpose of isolating single plaques representing a single peptide responsible
6 for the internalization of the phage. The phage is then amplified and the phage DNA is isolated and
sequenced to determine the sequence of the DNA encoding the peptide presented on the surface of
the phage which was isolated by biopanning.

Where plasmid display library is used, random peptides are presented on the surface
of a plasmid according to U.S. Patent No. 5,338,665, incorporated herein by reference. The plasmid
display library is then utilized in a manner similar to the method employed for the phage display
library by techniques known to those skilled in the art.

After determining the sequence of the peptides isolated by the biopanning (whether
by phage or plasmid display libraries or any other technique known to those skilled in the art),
“free” peptides (peptides without phage) may be synthesized according to peptide synthesis methods
16 (e.g. Merrifield solid phase synthesis). Such peptides are then conjugated to cargo. In a preferred
embodiment, the peptides are synthesized such that they are biotinylated and may be conjugated to
avidin labeled cargo (e.g. avidin β -gal, avidin Cy3). This allows for ease of screening of multiple
peptides for their ability to internalize cargo. Additionally, the peptide may be expressed as a fusion
protein with the cargo of interest (e.g. β -gal) by methods known to those skilled in the art. See, e.g.,
21 Villaverde et al., 1998, *Biotechnology and Bioengineering* 59:294-301.

1 Other preferred cargo include, but are not limited to, proteins, such as suicide proteins (*e.g.* HSV TK), tumor suppressor proteins, transcription factors, kinase inhibitors, kinases, apoptotic proteins, anti-apoptotic proteins, cell cycle regulatory proteins, viral and cellular antigens, toxins, transgenes (encoding for, *inter alia*, protein, RNA, ribozymes, antisense RNA), RNA, plasmids, oligonucleotides (single and double stranded) and virus.

6 The peptide conjugates (peptide + cargo) are then incubated with a target cell to allow for delivery of the peptide-cargo complex into the cell (*e.g.* Hig-82 cells). The ability of the peptide to transfer the cargo into the target cell may be measured by the presence of the cargo in the target cell by techniques known in the art. Where the cargo is β -gal, the addition of Xgal to the cells will produce a blue color in the cells if the β -gal is present. Where the cargo is Cy3, confocal microscopy may be employed to determine whether the cells fluoresce. Functional assays may also determine the presence of cargo in a cell. For example, but not by way of limitation, where the cargo is CFTR (or a nucleic acid encoding CFTR), the manifestation of a functional chloride ion channel would indicate delivery of the CFTR cargo to the target cell. Where the cargo is a toxin, cell death may indicate the presence of the cargo in the target cell and, where the cargo is a virus (*e.g.* Human Immunodeficiency Virus, Murine Leukemia Virus, Equine Infections Anemia Virus), the virus may comprise green fluorescent protein (GFP) as a marker or the virus may be labeled with Cy3, also a fluorescent marker to track the internalization of the virus by the peptides of the present invention in cells which would otherwise be resistant to infection by the virus. If the virus is a viral vector comprising a transgene, the presence of the virus in the cell may be demonstrated by the presence of a transgene product. The presence of the cargo in the nuclei by the methods described above,

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1 indicates that the peptides are capable of facilitating the translocation of the cargo to the nucleus and
may be demonstrated as described for internalization generally. For example, confocal microscopy
may be used to demonstrate the presence of a fluorescently tagged molecule in the nucleus.
Alternatively, the cells may be harvested and the nuclei separated therefrom for the determination
of the presence of a functional cargo therein by methods known to those skilled in the art..

6 In accordance with the present invention, screening for internalizing peptides by
phage biopanning yielded the peptides represented by SEQ ID NO:1 through SEQ ID NO:18,
further illustrated in Table 1 above. Particularly preferred peptides include KRIHPRLTRSIR (SEQ
ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRKLRRLK (SEQ ID NO:4) and
RRQRRTSKLMKR (SEQ ID NO:5) which facilitated the internalization of phage as well as the
facilitation of the internalization of a cargo (e.g. β -gal and Cy3).

16 The peptides of the present invention may also be useful for the determination of the
cell proteins which mediate internalization. For example, a cell lysate may be prepared from the
cells used to isolate the internalizing peptide. The internalizing peptide may be fused to a
polypeptide (e.g. glutathione-S-transferase or poly-histidine) which can be used for immuno-affinity
purification. The peptide fusion can then be incubated with the cellular lysate and passed over a
column specific for the fusion peptide (e.g. a glutathione column for the glutathione-S-transferase
fusion or a nickel or cobalt column for the poly-his fusion). Proteins which bind to the internalizing
peptide (e.g. cell surface receptors) may remain bound to the peptide fusion during the purification
process and be purified along with the peptide fusion. The peptide-bound protein may then be
21 isolated and its sequence may be determined by methods known in the art (e.g. N-terminal protein

1 sequencing). Such determination may lead to the identification of other pathways which might be
useful for the delivery of cargo to a target cell.

The peptides of the present invention can be prepared by classical methods known
in the art, for example, by using standard solid phase techniques. The standard methods include
exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation,
6 classical solution synthesis, and recombinant DNA technology. See, *e.g.*, Merrifield, 1963, *J. Am.*
Chem. Soc. 85:2149, incorporated herein by reference.

On solid phase, the synthesis is typically commenced from the C-terminal end of the
peptide using an alpha-amino protected resin. A suitable starting material can be prepared, for
instance, by attaching the required alpha-amino acid to a chloroethylated resin, a hydroxymethyl
resin, or a benzhydrylamine resin. One such chloromethylated resin is sold under the tradename
BIO-BEADS SX-1 by Bio Rad Laboratories, Richmond, CA, and the preparation of the
hydroxymethyl resin is described by Bodonsky et al., 1966, *Chem. Ind.* 38:1597. The
benzhydrylamine (BHA) resin has been described by Pietta and Marshall, 1970, *Chem. Commn.* 650
and is commercially available from Beckman Instruments, Inc., Palo Alto, CA, in the hydrochloride
16 form.

Thus, the peptides of the invention can be prepared by coupling an alpha-amino
protected amino acid to the chloromethylated resin with the aid of, for example, cesium bicarbonate
catalyst, according to the method described by Gisin, 1973, *Helv. Chim. Acta.* 56:1467. After the
initial coupling, the alpha-amino protecting group is removed by a choice of reagents including
21 trifluoro acetic acid (TFA) or hydrochloric acid (HCl) solutions in organic solvents at room

1 temperature.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups urethane type protecting groups, aliphatic urethane protecting groups and alkyl type protecting groups. The side chain protecting group remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling. The side chain protecting group must be removable upon the completion of the synthesis of the final peptide and under reaction conditions that will not alter the target peptide.

After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the desired order. An excess of each protected amino acid is generally used with an appropriate carboxyl group activator.

After the desired amino acid sequence has been completed, the desired peptide is decoupled from the resin support by treatment with a reagent such as TFA or hydrogen fluoride (HF), which not only cleaves the peptide from the resin, but also cleaves all remaining side chain protecting groups.

These solid phase peptide synthesis procedures are well known in the art and are further described by Stewart and Young, *Solid Phase Synthesis* (2nd Ed., Pierce Chemical Co., 1984), incorporated herein by reference.

The internalizing peptides of the present invention may be synthesized with additional groups, such as biotin or other markers, such that the peptide may be tracked in the cell or conjugated via the additional group to cargo. The peptides may also be later modified to incorporate

1 any desired additional groups according to methods known in the art.

The internalizing peptides are typically synthesized as the free acid but could be readily prepared as the amide or ester where desired. Other types of modifications include, but are not limited to, methylation, acetylation and adding a benzyloxycarbonyl (t-BOC) group. Additionally the peptides may be synthesized as cyclic peptides. The C-terminal carboxyl group or
6 a C-terminal ester can be induced to cyclize by internal displacement of the -OH or ester of the carboxyl group or ester respectively with the N-terminal amino group to form a cyclic peptide. Such methods are well known in the art.

Cyclization of the peptides or incorporation of a desamino or descarboxy residue at the termini of the peptides of the present invention, so that there is no terminal amino or carboxy group, to decrease susceptibility to proteases or to restrict the confirmation of the peptide, are also contemplated by the present invention.

The present invention also provides for compositions comprising the internalizing peptides of the present invention, complexes comprising the peptides linked to cargo, and immunogens of the present invention. Non-limiting examples include: the administration of
16 internalizing peptides and peptide-cargo *in vivo* by oral, pulmonary, parenteral (intramuscular, intra-articular, intraperitoneal, intravenous (IV) or subcutaneous injection), inhalation (via a fine powder formulation or a fine mist), transdermal, nasal, vaginal, rectal, or sublingual routes of administration and can be formulated in dosage forms appropriate for each route of administration.

The peptide-cargo complexes of the present invention may be administered with a
21 carrier. Such carriers include any suitable physiological solution or dispersant or the like. The

1 physiological solutions include any acceptable solution or dispersion media, such as saline or
buffered saline. The carrier may also include antibacterial and antifungal agents, isotonic and
adsorption delaying agents, and the like. Except insofar as any conventional media, carrier or agent
is incompatible with the active ingredient, its use in the compositions is contemplated.

6 The invention is further directed to methods for using the compositions of the
invention for *in vivo* or *ex vivo* applications in which it is desired to deliver cargo into cells to
achieve a particular phenotypic effect. *In vivo* applications involve, *e.g.*, the direct administration
of the peptide-cargo complex of the present invention formulated as a composition to the cells of an
individual. *Ex vivo* applications involve, *e.g.*, the transfer of the peptide-cargo complex of the
present invention directly to autologous cells which are maintained *in vitro*, followed by the re-
administration of the cells comprising the internalized cargo to a recipient.

16 Dosage of the peptide-cargo complex of the present invention to be administered *in*
vivo in order to effect efficient delivery of cargo into a target cell and/or achieve a phenotypic effect
correlated to the delivery of cargo is determined with reference to various parameters, including the
species of the subject, the age, weight, and disease status and the particular physiological conditions
requiring phenotypic alteration. Dosage also depends upon the location of the cells to be targeted
within the subject. For example, target cells of the lung may require different dosages than
administration into the blood stream of an organism. The dosage is preferably chosen so that
administration causes an effective result, as measured by molecular assays or phenotypic alteration.
Such assays include Western blot of a particular protein being administered or encoded by a transgene
21 that has been administered, immunoprecipitation, immunocytochemistry, or other techniques known

1 to those skilled in the art. Dosages may range from 0.01 nM to 1 μ M. In a preferred embodiment, the dosage ranges from 1 nM to 1 μ M. In a particularly preferred embodiment, the dosage is 1.5 nM for pep4 and pep5 and 15 nM for pep2 and pep3.

6 The practice of the present invention can be achieved by employing a number of conventional techniques of molecular biology, microbiology, recombinant DNA technology, biochemistry and immunology which are within the skill of the art. Such techniques are explained fully in the literature, see, e.g., Fritsch and Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd Edition (1989); Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, (1992); incorporated herein by reference.

Although only preferred embodiments of the invention are specifically described above, it will be appreciated that modifications and variations of the invention are possible without departing from the spirit and intended scope of the invention.

The following examples are provided to more clearly illustrate the aspects of the invention and are not intended to limit the scope of the invention.

EXAMPLES

Example 1: Titering M13 Phage

16 A phage display library (Ph.D.-12™ Catalog # 8110) was obtained from New England BioLabs (Beverly, MA). The Ph.D.-12™ phage display library is a library of M13 coliphage with each phage displaying a different 12 residue peptide and represents 1.9×10^9 independent clones. The randomized peptides in the library are expressed between the leader
21 sequence and the N-terminus of the minor coat protein pIII, resulting in an average valency of 5

1 displayed peptides per virion. The display vector for the library is a derivative of wild-type M13 phage which is not a lytic phage. There is a physical linkage between each displayed peptide and its encoding DNA for easy determination of the selected peptide sequence.

E.coli ER2537 was the host strain used for the M13 phage display library.

ER2537 is a robust F+ strain with a rapid growth rate and is well suited for M13 propagation.

6 For titering the phage, ER2537 was streaked out from a glycerol stock onto a minimal plate (500 ml 2X M9 salts (12 g Na₂HPO₄, 6 g KH₂PO₄, 1 g NaCl, 2 g NH₄Cl per liter), 500 ml 3% agar, 20 ml 20% glucose, 2 ml 1 M MgSO₄, 0.1 ml 1 M CaCl₂, 1 ml thiamine (10 mg/ml)) for phage titering. After 24 hours, a single colony was picked and used to inoculate 5 ml of LB (10 g bacto tryptone, 5 g yeast extract, and 5 g NaCl per liter) which was grown for 3 hours to mid-log phase (OD₆₀₀ ~ 0.5) at 37° C. Approximately 200 µl of the stock was then spread onto a plate containing LB, IPTG and Xgal (per liter LB add 15 g agar, 0.05 g IPTG and 0.04 g Xgal). The plates were inoculated with serial dilutions of 10-fold of the phage stock and incubated overnight at 37° C. The cells infected with phage stained blue due to the presence of the phage (which contains β-gal) and the plaques were counted to determine the titer. The titer was preferably 1-2 x 10¹¹. Biopanning (as described below in Example 2) can be carried out with as little as 10⁹ plaque forming unites (pfu).

Example 2: Screening a Phage Display Library to Identify Internalizing Peptides:

Hig-82 biopanning: Hig-82 cells (rabbit synovial cell line supplied by Christopher Evans, University of Pittsburgh, ATCC Deposit No. CRL-1832) were employed for screening the New England Biolabs Ph.D-12™ phage-display library. The Hig-82 cells were cultured in

1 10 cm plates and grown to 100% confluency. The cells were then incubated with approximately
4 x 10¹⁰ phage in a volume of 10µl overnight at 4 °C. The Hig-82 cells were then harvested and
washed twenty times with wash buffer (25 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM CaCl₂,
10 mM MgCl₂, 1% bovine serum albumin (BSA)). The last washing solution was collected and
titered to determine if any phage were present. This wash had no phage indicating that the
6 washing was sufficient. Phage which were bound to the cells were eluted with 50 mM glycine,
pH 2.2 for 30 minutes at room temperature and the eluate was immediately thereafter neutralized
for two minutes with 0.2M NaPO₄ buffer, pH 8.0. The eluate was collected for phage titering
and amplifying.

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The cells were then trypsinized from the plate with 0.05% trypsin, 0.53 mM
EDTA and lysed by three consecutive rounds of freeze/thaw in dry ice/EtOH. The lysed cells
were then centrifuged and the supernatant was collected for phage titering and amplification.
The cell pellets containing the cell debris were washed with wash buffer (see above) five times
and the last wash was collected for phage titering. The cell pellet containing the cell debris was
then eluted with 50 mM glycine, pH 2.2 for 30 minutes at room temperature and the eluate was
16 immediately thereafter neutralized for two minutes with 0.2 M NaPO₄ buffer, pH 8.0. The eluate
was collected and saved for phage titering and amplification.

Phage titering was accomplished as described above in Example 1 and the phage
were amplified by adding the eluates to a 20 ml ER2537 culture grown to early-log phase in LB
medium as described above in Example 1 and incubating for 4.5 hours at 37 °C with vigorous
21 shaking. The culture was then centrifuged for 10 minutes at 10,000 rpm in a Sorvall model SS-

1 34 centrifuge at 4 °C. The supernatant was transferred to a new tube and spun a second time.
 The upper 80% of the supernatant was then transferred to a new tube and 1/6 volume of
 PEG/NaCl (20% w/v polyethylene glycol-8000, 2.5 M NaCl) was added and incubated overnight
 at 4 °C to precipitate the amplified phage. The PEG precipitate was then centrifuged for 15
 minutes at 10,000 rpm at 4 °C (supernatant was decanted and pellet was respun briefly) and
 6 residual supernatant was removed with a pipette. The pellet was resuspended in 1 ml TBS (50
 mM Tris-HCL (pH 7.5), 150 mM NaCl) and spun in a microcentrifuge tube to remove any
 remaining debris. The supernatant was transferred to a fresh microcentrifuge tube and re-
 precipitated with 1/6 volume PEG/NaCl, incubated for 60 minutes on ice and microcentrifuged
 for 10 minutes at 4 °C. The pellet was resuspended in 200 µl TBS, 0.02% NaN₃ and
 recentrifuged to remove any remaining debris. The supernatant represented the amplified phage.

The procedure (referred to hereafter as biopanning) was repeated a total of three
 times to achieve phage stocks enriched for phage which were internalized into the Hig-82 cells.

Human Primary T-cell biopanning: Human primary CD4⁺ and CD8⁺ T-cells (purified
 from peripheral blood mononuclear cells (PBMC) of normal donors using immunomagnetics
 beads (Miltenyi Biotech, Bergish Gladbach, Germany)) were employed for screening the New
 England Biolabs Ph.D-12™ phage-display library. The T-cells were incubated at 37°C
 overnight in the presence of 25 IU/ml interleukin2 (IL2). The cells were then incubated with
 approximately 4×10^{10} phage in a volume of 10µl for 4 hours at 4 °C with gentle shaking. The
 T-cells were then harvested and washed extensively with tris-buffered saline (TBS). Phage
 21 which were bound to the cells were eluted with 50 mM glycine, pH 2.2 for 10 minutes at room

1 temperature and the eluate was immediately thereafter neutralized for two minutes with 0.2M
NaPO₄ buffer, pH 8.0. The eluate was collected for phage titering and amplifying.

The cells were then trypsinized from the plate with 0.05% trypsin, 0.53 mM
EDTA, washed 2x with TBS at room temperature, centrifuged to remove wash and resuspended
in 0.2 ml TBS. The T-cells were then lysed by three consecutive rounds of freeze/thaw in dry
6 ice/EtOH. The lysed cells were then centrifuged and the supernatant was collected for phage
titering and amplification.

Phage titering was accomplished as described above in Example 1 and the phage
were amplified as described for Hig-82 cells.

The procedure (referred to hereafter as biopanning) was repeated a total of three
times to achieve phage stocks enriched for phage which were internalized into the T-cells.

Calu 3 cell biopanning: The human lung adenocarcinoma cell line Calu 3 (ATCC,
Rockville, MD.) was cultured in a flask with a 1:1 ratio of DMEM media and F12 media to 70%
confluency, then trypsinized from the flask, washed 1x with TBS and transferred into a cell
culture filter and grown to 100% confluency in a 1:1 ratio of DMEM media and F12 media.

16 Phage biopanning was performed as above for human primary T-cells.

Cervical Tissue biopanning: Surgically resected cervical mucosa cells from human
patients were grown in a 60 mm tissue culture dish in the presence of 5 ml of complete DMEM
media. The cervical mucosa cells were then incubated with approximately 4×10^{10} phage in a
tissue culture dish for 18 hours. The mucosa tissue was then trypsinized and class II positive
21 cells were selected from a single cell suspension using immunomagnetics beads (Miltenyi

1 Biotech, Bergish Gladbach, Germany) following the manufacturer's protocol. The purified
mucosal cells were then lysed by three consecutive rounds of freeze/thaw in a -70°C freezer.
The lysed cells were then centrifuged and the supernatant was collected for phage titering and
amplification.

Phage titering was accomplished as described above in Example 1 and the phage
6 were amplified as described for Hig-82 cells.

Example 3: Identification of phage displayed peptides which were internalized into Hig-82 cells,
T-cells, Calu3 cells, and Cervical Tissue.

After three rounds of biopanning, the enriched phage preparations were plaqued
as described above in Example 1 for phage titering. A single plaque was then picked (from
plated containing approximately 100 plaques) with a sterile wooden stick and transferred to a
tube containing 1 ml of ER2537 culture in LB and incubated for 4.5 hours with shaking. The
phage were amplified as described above in Example 2. Phage DNA was prepared from the
amplified stock by centrifuging the 1 ml cultures in a microcentrifuge for 30 seconds, removing
the supernatant, adding 200 µl PEG/NaCl and precipitating the phage for 10 minutes at room
16 temperature. The precipitated phage were then centrifuged for 10 minutes in a microcentrifuge
and the supernatant was discarded (a subsequent spin was performed to remove any remaining
supernatant). The pellet was resuspended in 100 µl iodide buffer (10 mM Tris-HCl (pH 8.0), 1
mM EDTA, 4 M NaCl), 250 µl EtOH was added and the mixture was incubated for 10 minutes
at room temperature to preferentially precipitate single-stranded phage DNA and leave most of
21 the phage protein in solution. The precipitated phage DNA was then centrifuged for 10 minutes

1 in a microcentrifuge and the pellet was washed with 70% EtOH and dried briefly under vacuum.
The dried phage DNA pellet was then resuspended in 30 μ l TE buffer (10 mM Tris-HCl (pH 8.0,
1 mM EDTA). The phage DNA (approximately 5 μ l of the 30 μ l preparation) was then
sequenced (automated DNA sequencing at the University of Pittsburgh) to determine the
sequence of the peptides which were internalized. Figure 7 shows the uptake of M13 phage
6 labeled with Cy3 by peptide 3 (SEQ ID NO:3) and peptide 5 (SEQ ID NO:5). The peptides
identified are pep 1 through pep 66 (SEQ ID Nos:1-18 and 25-72 respectively) as represented in
Table 1-4 above.

To show that the peptides of the present invention are able to facilitate
internalization and transport of protein complexes and phage (virus) to the nucleus of a cell,
biotinylated peptides were coupled to streptavidin-488. See Bayer et al., *Histochem. Cytochem.*
24:933-939 (1976); Ivanenkov & Menon, *Biochim. Biophys. Acta.* 1448:463-472 (1999). The
use of the 488 fluorescent marker allows for analysis of the treated cells by confocal microscopy
to determine the exact location of the complexes in the cells. Peptide 5 (SEQ ID NO:5) was able
to facilitate the efficient internalization of the streptavidin-488 complex (Figure 13A) with a
16 significant percentage of the peptide-streptavidin-488 complex being found in the nucleus by
confocal microscopy of treated cells (Figure 13B).

To determine if the peptides of the present invention are able to facilitate
internalization of intact M13 phage(a virus), as suggested by the screening and isolation
procedure, a peptide 5/M13 phage complex was labeled with Cy3 (as for Figure 5) and added to
21 rabbit synovial fibroblasts. Labeled peptide 5/M13 phage complex was detected in the treated

1 cells (Figure 13D) compared to the control phage (Figure 13C). Thus peptide 5, as well as
peptides 2, 3, and 4 (SEQ ID NOs:2-4 respectively) were able to facilitate internalization and
nuclear localization of protein complexes as well as intact M13 phage. Therefore, the peptides of
the present invention are useful for facilitating the internalization of virus and viral vectors.

6 Example 4: Identifying peptides which facilitate internalization of conjugated cargo (β -
galactosidase and Cy3)

Internalizing peptides 1-6 (SEQ ID Nos:1-6 respectively), which were identified
as described above in Example 3, were synthesized, and biotinylated (synthesis and biotinylation
performed by the Biotech Center of the University of Pittsburgh). The biotinylated peptides
were then conjugated to avidin β -gal or avidin Cy3 at room temperature for 2 hours.

The peptide conjugates were then added to cells (Hig-82 cells, rabbit synovial
cells, human synovial cells, rabbit synovial lining, human primary airway cells HBE 144, human
primary islet cells, murine myoblast cells C2C12, dog kidney epithelial cells MDCK, murine
fibroblast cells NIH3T3 and murine tumor cells MCA 205 (human synovial cells and human
16 primary airway cells (HBE144) were isolated from patients from the Presbyterian Hospital,
University of Pittsburgh by standard techniques for establishing a primary cell culture; human
islet cells were provided by the University of Miami and were isolated by standard techniques for
establishing a primary cell culture; C2C12, MDCK, 3T3 and MCA205 cells were purchased
from ATCC, Bethesda, Md.; all cells were cultured in DMEM and grown to 100% confluency)
21 and incubated while gently rotating at 37 °C for 3 hours with TBS buffer. The cells were then

1 washed 10 times with TBS buffer and fixed with 4% paraformaldehyde at room temperature for
 30 minutes. Cells were then washed 3 times with TBS buffer and stained with 1 mg/ml X-gal
 (Boehringer Mannheim, Indianapolis) at 37°C overnight for β -gal-conjugated peptides. Cells
 which were blue indicated the presence of β -gal in the cells. For Cy3-conjugated peptides,
 fluorescence of the cells as measured by confocal microscopy indicated the ability of the peptide
 6 to internalize the Cy3 (a small molecule cargo).

Figure 1A shows the results of peptide- β -gal complex internalization into Hig-82
 cells using peptide 1, 2, 3, 4, 5 and 6 (SEQ ID Nos:1-6 respectively) as compared to
 antennapedia peptide (RQIKIWFQNRRMKWKK; SEQ ID NO:19), TAT-PTD
 (YGRKKRRQRRR; SEQ ID NO:21), and a random control peptide (ARPLEHGSDKAT; SEQ
 ID NO:20). Figure 1B shows the relative strength of internalization mediated by peptides 2, 3, 4
 and 5 (SEQ ID NOs:2-5 respectively) as compared to antennapedia peptide (SEQ ID NO:19),
 TAT-PTD (SEQ ID NO:21), and a random control peptide (SEQ ID NO:20). In Figure 1B,
 various concentrations of peptides linked to β -gal were tested (1:1 = 150 nM of β -gal). The
 results show that peptides 4 and 5 (SEQ ID NOs:4 and 5 respectively) effect internalization of β -
 16 gal linked thereto at a concentration equal to or less than TAT-PTD, suggesting that
 internalization mediated by peptides 4 and 5 is equal or superior to internalization mediated by
 TAT-PTD. Peptides 3 and 4 achieved internalization of β -gal at a slightly higher concentration.
 Strikingly, peptides 2-5 were more effective than the commercially available antennapedia
 peptide.

21 Figure 2 A-D shows the ability of peptide 1 (SEQ ID NO:1) (Figure 2A and 2C,

1 low and high magnification respectively) and peptide 3 (SEQ ID NO:3) (Figure 2B and 2D, low
and high magnification respectively) to facilitate the internalization of β -gal into rabbit synovial
cells. Figure 3A-D shows the ability of peptide 5 (SEQ ID NO:5) (Figure 3A and 3B, high and
low magnification respectively) and peptide 1 (SEQ ID NO:1) (Figure 3C and 3D, high and low
magnification respectively) to facilitate the internalization of β -gal into human synovial cells.

6 Figure 4 shows the ability of peptides 2- 5 (SEQ ID NOs:2-5, respectively), TAT-
PTD (SEQ ID NO:21), antennapedia peptide (SEQ ID NO:19), a random control peptide (SEQ
ID NO:20) and an adenoviral vector encoding β -gal (Ψ 5) to facilitate *in vivo* transfer of peptide-
conjugated β -gal into rabbit synovial lining. Peptide- β -gal complexes (fusions) were injected
into rabbit knee synovial lining, the lining was then removed, washed with wash buffer, fixed
with 4% paraformaldehyde, and stained with X-gal. Peptides 4, 5 and TAT-PTD showed the
highest level of uptake into the rabbit synovial lining (Figure 4A). The control peptide and saline
alone showed no uptake. The level of uptake was significantly higher for all the internalizing
peptides as compared to the adenoviral vector encoding β -gal, which was injected 3 days prior to
injection of the peptide- β -gal constructs (Figure 4A).

16 Figure 4B shows the histological analysis of the rabbit synovial lining using an
eosin counter-stain. The histological analysis showed that β -gal staining was intracellular and
limited to the synovial lining. Peptide 5 (SEQ ID NO:5) was able to facilitate uptake of β -gal in
nearly 100% of the synovial cells *in vivo* (Figure 4B). Injection of peptide 5 (SEQ ID NO:5) into
day 14 murine tumors which were prepared by subcutaneously injecting MCA-205 cells
21 (fibrosarcoma cell line) resulted in significant β -gal staining that was also significantly higher

1 than that observed for an adenoviral vector encoding β -gal. These *in vivo* results show that the peptides of the present invention can facilitate efficient internalization of protein complexes into joints and tumors and thus are useful for delivery of proteins of interest (such as apoptotic proteins, suicide proteins, tumor suppressor proteins, chemotherapeutic agents, etc) to joints (*e.g.* arthritic joints) and tumor cells.

6 Figure 6 A-I shows the ability of peptide 5 (SEQ ID NO:5) to facilitate the uptake of β -gal in (6A) HIG-82 cells; (6B) rabbit primary synovial cells; (6C) human primary synovial cells; (6D) HBE 144 primary human airway epithelial cells; (6E) MDCK polarized canine kidney cells ; (6F) human β islet primary cells; (6G) C2C12 murine myoblast cells; (6H) MCA205 murine fibrosarcoma cells; and (6I) NIH3T3 cells. Additionally, Figure 9B-C shows the ability of peptide 5 to facilitate internalization of eGFP in human islets at low magnification (9B is a photomicrograph of the histological ly stained cells and 9C shows the fluorescent detection of eGFP). Figure 9D-E show the ability of peptide 5 to facilitate internalization eGFP in human islets at high magnification (9D is a photomicrograph of the histological ly stained cells and 9E shows the fluorescent detection of eGFP). Figure 9F-G shows the ability of peptide 5 to facilitate the internalization of eGFP in human dendritic cells (9F is a photomicrograph of the histological ly stained cells and 9G shows the fluorescent detection of eGFP). Figure 9A is a schematic representation of the expression construct encoding the peptide5-eGFP fusion protein.

21 Figure 17 shows the ability of peptide-5 linked to β -gal to transduce CD34+/LIN- stem cells. Peptide 5 is biotin labeled and the β -gal is avidin labeled so that they may be linked together by an avidin/biotin interaction. The cells which stained dark were transduced with the

1 peptide/ β -gal complex as indicated by an arrow.

To make the nucleic acid encoding the peptide-eGFP fusion depicted in Figure 9A, PCR was employed using 5' and 3' primers that encoded the peptide and a his tag, respectively, and had complimentary sequences to eGFP DNA coding sequences. The resultant PCR product was subcloned into the bacterial expression vector pET (Novagen, Madison, WI).

6 For protein expression, the expression vector comprising the DNA encoding a peptide eGFP fusion with a his tag was transfected into bacterial cells which were grown by standard techniques. The cells were induced to express the fusion product with 1 mM IPTG and were harvested and lysed. The fusion protein was purified over a Nickel column. The purified protein was then added to the media of Hig-82 cells in culture. The eGFP positive cells were visualized under a fluorescence microscope. Figure 8 shows the ability of peptide 3 (SEQ ID NO:3) and peptide 5 (SEQ ID NO:5), as well as the antennapedia peptide (SEQ ID NO:19) to facilitate the internalization of β -gal into murine tumor cells *in vivo*. MCA 205 tumor cells (5×10^5 cells) were injected subcutaneously into the flank of a C57/BL6 mouse. At day 14, a single intra-tumoral injection of the peptide β -gal complex was performed. The mice were sacrificed 3 hours post-
16 injection and the tumor tissue was isolated, sectioned and stained with X-gal.

These data indicate that the internalizing peptides of the present invention can facilitate the uptake of cargo (β -gal or Cy3) into cells both *in vitro* and *in vivo*. Additionally, since the cargo was transported to the nucleus of the cells, the data also indicate that the peptides facilitated nuclear translocation of the cargo as well (see Figures 1, 2, 3, and 6).

21 Example 5: Peptide competition assay

1 A peptide competition assay was carried out to determine the relative efficiency
and specificity of the various peptides. The peptides were conjugated to β -gal as described
above in Example 4 and incubated with Hig-82 cells grown to 85% confluency in 24 well plates
in the presence of non biotinylated peptides for 3 hours at 37 °C in TBS buffer while being
gently rotated. The cells were then washed 10 times with TBS buffer, fixed with 4%
6 paraformaldehyde at room temperature for 30 minutes, washed 3 times with TBS buffer, and
stained with Xgal, as described above in Example 4, overnight. Cells which stained blue had
internalized peptide-conjugated β -gal.

Figure 5 shows the ability of the various non-biotinylated peptides to compete for
internalization of the peptide-conjugated β -gal. In rows 1-4 peptide-conjugated β -gal comprising
peptide 2, 3, 4 and 5 respectively (SEQ ID Nos: 2-5 respectively) were incubated with cells in
the presence of non biotinylated peptides 1-6 (SEQ ID Nos:1-6 respectively) which were added
to columns 1-6 respectively. Peptide 1 (SEQ ID NO:1) did not block the internalization by any
of the peptides. Peptides 2-6 (SEQ ID Nos:2-6 respectively) blocked the internalization of
peptide 2-conjugated β -gal and peptides 4 (SEQ ID NO:4) and peptide 5 (SEQ ID NO:5) blocked
16 the internalization of peptide 2 through peptide 6-conjugated β -gal indicating that these peptides
more efficiently bound to the cells than any others.

Figure 12 also shows the ability of the various non-biotinylated peptides to
compete for internalization of the peptide-conjugated β -gal. A 100 fold excess of peptide 5
(SEQ ID NO:5) was able to completely inhibit the uptake of peptides 3, 4 (SEQ ID NOs:3 and 4
21 respectively) and the antennapedia peptide (SEQ ID NO:19) and significantly inhibit the uptake

1 of TAT-PTD (SEQ ID NO:21) and itself. Surprisingly peptide 6 (SEQ ID NO:6) was able to
 inhibit the uptake of peptide 2 and 3, but not peptides 4 and 5, even though peptide 6 is only
 weakly able to transduce cells compared with peptides 2-5. Also tested were saline alone, a
 random peptide (SEQ ID NO:20), TAT-PTD (SEQ ID NO:21) and a polylysine peptide having a
 MW between 1,000 D and 4,000 D with an average MW of 2,500 D (purchased from Sigma, St.
 6 Louis, MO). The ability of the polylysine peptide to inhibit uptake suggests that the charge of
 the peptide is important for at least part of the process of peptide-mediated internalization.

Example 6: Circular Dichroism Spectroscopy:

Stock solutions of peptides 1, 2, 3, 4, 5 and 6 (SEQ ID NOs:1-6 respectively), as
 well as TAT-PTD (SEQ ID NO:21), and the antennapedia peptide (SEQ ID NO:19) were diluted
 to 1.5 mg/ml in 5 mM KPO₄ at pH 7.4. Measurements were made using an Aviv 62DS
 spectropolarimeter over the wavelength range from 300 to 190 nm (except for TAT-PTD, where
 sample absorbency precluded measurements below 192 nm even after sample dilution), with a
 0.5 nm step size, at room temperature using a 0.1 cm pathlength quartz cell (Hellma). Ellipticity
 measurements below 190 nm were precluded due to solvent absorption. All reported spectra were
 16 baseline corrected (by subtraction of similarly collected, averaged baselines of buffer alone) and
 smoothed using a Savitzky Golay filter.

Figure 10A-H shows that the peptides have a small dip at a wavelength of 225
 nm which indicative of possible insignificant secondary structure for α helices.. Specifically,
 Figure 10 A-H shows the circular dichroism plot for peptide 1 (SEQ ID NO:1; Figure 10A),
 21 peptide 2 (SEQ ID NO:2; Figure 10B), peptide 3 (SEQ ID NO:3; Figure 10C), peptide 4 (SEQ

1 ID NO:4; Figure 10D), peptide 5 (SEQ ID NO:5; Figure 10E), peptide 6 (SEQ ID NO:6; Figure
10F), antennapedia peptide (SEQ ID NO:19; Figure 10G) and a random peptide (SEQ ID NO:20;
Figure 10H).

Figure 11A & B shows that qualitatively, the spectra of peptides 1-6 fall into three
general groups. Figure 11A shows that the spectra of the highly active peptides 4 and 5 (SEQ ID
6 NOs: 4 and 5 respectively) are nearly super-imposable with that of TAT-PTD (SEQ ID NO:21)
and somewhat similar to the antennapedia peptide (SEQ ID NO:19). Peptides 2 and 3 (SEQ ID
NOs: 2 and 3 respectively), which have an intermediate activity, yield spectra which are similar
to each other, but significantly different from peptides 4 and 5. The lower activity peptides 1 and
6 (SEQ ID NOs: 1 and 6 respectively) fall into a third class and share some similarity to the
random peptide (SEQ ID NO:20) which does not have activity. The peptides do not have a
significant helical content, which would give rise to a bilobed minima at ~205 and 220 nm, and a
large positive peak at ~195 nm. Rather, the peptides appear to be enriched in a poly-proline-type
helix (which does not require the presence of prolines). *See Sreerama & Woody, Biochemistry*
33:10022-10025 (1994). This type of structure is consistent with some of the observations made
16 regarding the antennapedia peptide where the substitution of the prolines into the peptide
sequence did not disrupt activity, nor did the substitution of D-amino acids and/or reversal of the
chain direction disrupt activity. *See Derossi et al., J. Biochem.* 217:18188-18193 (1996); Berlose
et al., *Eur. J. Biochem.* 242:372 (1996). These substitutions would not affect the charge
distribution on a poly-proline-type helix, nor be disruptive to this structure. The amphipathic
21 cationic peptides of the present invention likely interact with the negative charges on the surface

1 of the bilayers.

The CD spectra of the peptides was also analyzed following addition to small unimellar vesicles (SUVs). In the presence of SUVs, which comprise mixed phospholipids that are primarily phosphatidylcholines, no significant changes were observed in the CD spectra. However, when the SUVs were composed of dipalmitoyl phosphatidylserine, a large change in the resulting CD spectra was observed which correlated with transfer activity.

Example 7: Delivery of an Apoptotic Peptide to Cells via the Peptides of the Present Invention:

To demonstrate that the peptides of the present invention could facilitate the uptake of an apoptotic peptide KLAKLAKKLAKLAK (SEQ ID NO:23) into cells and induce apoptosis therein, a continuous peptide, termed the "death peptide", comprising peptide 5 and the apoptotic peptide was synthesized (RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24)) (Research Genetics, Huntsville, AL).

2 x 10⁵ cells (HIG82, MCA205 or Jurkatt) were seeded into 24 well plates. Forty-eight hours later, 100 µM, 50 µM, 25 µM, 6.25 µM and 3.1 µM of the either the death peptide (SEQ ID NO:24), peptide 5 alone (SEQ ID NO:5) or the apoptotic peptide alone (SEQ ID NO:23) was added to the cells. To check for cell viability, three hours later, the media was aspirated and 1 ml of serum-free media containing 0.25 mg of MTT was added to each well and incubated for 4 hours at 37°C. MTT-containing media was then removed and 1 ml of absolute isopropanol containing 0.4N HCl was added. Photographs were taken of the plates, then the cells were harvested and any debris was centrifuged. OD was taken at $\lambda = 570$ nm. Each sample point was performed in triplicate.

1 Figure 14 and Figure 15 shows the ability of the "death peptide" to impair HIG-82
cell viability. In contrast, peptide 5 alone and the apoptotic peptide alone did not impair
viability.

To determine whether the death peptide could induce apoptosis in tumor cells and
inhibit tumor growth *in vivo*, C57BL/6 mice bearing day 7 tumors in each flask (seeded with 1 x
6 10⁵ MCA205 cells) were injected daily for 10 days with a 50 µl volume of 1 mM death peptide
(SEQ ID NO:24; DP1) or the apoptotic peptide alone (SEQ ID NO:23; KLA) or a saline mock
into both tumors. Five mice were used in each group. Tumor volume was estimated by
multiplying maximum length x width². Separately, C57BL/6 with single, day 12 tumors were
injected with 1 mM death peptide (SEQ ID NO:24; DP1), apoptotic peptide alone (SEQ ID
NO:23; KLA) or saline for eleven days. Ten mice comprised each group. On the final day, the
mice were injected with the appropriate saline or peptide solution and sacrificed 3 hours post-
injection. Tumors were paraffin-embedded, sectioned, and stained for TUNEL and
counterstained with methyl green or stained with hematoxylin and eosin to reveal histologic
architecture.

16 As shown in Figure 16A, daily administration of 50 µl of 1mM death peptide
(SEQ ID NO:24; DP1) lead to a striking reduction or halt in tumor growth in the fibrosarcomas
compared to the apoptotic peptide alone (SEQ ID NO:23; KLA) or tris buffered saline (TBS).
By day 6, between the death peptide (DP1) and apoptotic peptide alone (KLA) groups, a p<=
0.026 by a two-tailed student's t-test of the means was observed, which became = 0.0001 by day
21 10. As a plot of individual tumor sizes shows (Figure 16D), both the death peptide (DP1) and

1 the apoptotic peptide alone (KLA) mouse groups begin with comparable tumor sizes. However,
by day 9 of treatment, no overlap exists in the tumor sizes of the DP1-treated cohort with KLA-
treated cohort. To test whether an immune response against the tumors may have been triggered
by the apoptosis, treatment was halted at day 14, however, in the DP1-treated mice. Tumors
continued to grow, directly correlating with the cessation of DP1 administration. Figure 16B
6 shows a comparison of tumor sizes between the DP1 and KLA-treated groups eight days
following the initiation of treatment. There is a dramatic reduction in tumor volume in the DP1-
treated mice, coincident with reduction in ulceration and bleeding in these mice. In a separate
experiment, 3 out of 10 mice with single flank tumors treated with DP1 had undetectable tumors
following 11 days of treatment. Importantly, repeated administration of DP1 resulted in no
obvious side-effects in treated mice.

To test whether apoptosis was the mechanism of tumor killing *in vivo* and to study
the effects of death peptide administration on tumor architecture, fibrosarcomas treated daily for
11 days with death peptide (SEQ ID NO:24; DP1), apoptotic peptide alone (SEQ ID NO:23;
KLA) or tris buffered saline (TBS) were injected with 50 µl of 1 mM DP1, KLA or TBS on the
eleventh day and sacrificed three hours later. Tumors were excised, embedded, sectioned, and
stained with either TUNEL or hematoxylin and eosin. As shown in Figure 16C, treatment with
DP1 mediates a potent apoptosis in MCA205 fibrosarcomas *in vivo*. A broad zone of anuclear
and acellular eosinophilic debris is ringed by a zone of tumor cells in the process of undergoing
apoptosis, as shown in the bottom right panel (Figure 16C). The zones of killing are substantial;
in some instances, an estimated 10-20% of total tumor volume stained TUNEL-positive

1 following DP1 administration. No TUNEL staining is observed in the KLA-treated tumors,
indicating the requirement for linkage to peptide 5 (SEQ ID NO:5) for induction of apoptosis.

In addition, the death peptide was administered to rabbits with IL-1-induced
arthritis (*see Ghivizzani et al. J. Immunol.* 159:3604 (1997)). Three arthritic rabbits received
the death peptide, three received peptide 5 alone and 3 received the apoptotic protein alone. The
6 rabbits were sacrificed 24 or 72 hours post-injection of the peptides and the rabbit knee capsules
were removed for histology analysis and TUNEL staining.

Figure 18 shows that the delivery of the death peptide (SEQ ID NO:24; DP1)
mediated apoptosis of hyperplastic synovium *in vivo* whereas the antimicrobial peptide alone
(SEQ ID NO:23; KLA) did not. Furthermore, injection of the death peptide (SEQ ID NO:24;
DP1) into arthritic rabbit joints caused a great reduction in the number of white blood cells in
the lavage fluid if IL-1 inflamed rabbit joints as compared to peptide 5 alone (SEQ ID NO:5;
peptide control) (*see* Figure 19). These data indicate that the internalizing peptides of the present
invention are effective for delivering apoptosis factors to arthritic joints and may be useful for
the treatment of arthritis.

16 To demonstrate that other apoptotic factors could effectively be delivered to cells,
the internalizing peptide 5 (SEQ ID NO:5; pep 5) of the present invention was linked to p53. An
expression clone was prepared by using PCR to first create an expression cassette having pep5 at
the amino terminus and a his tag at the carboxy terminus of the p53 coding sequence. The
expression cassette was cloned into the pet3b vector (Stratagene, La Jolla, CA). The expression
21 clone was then transformed into BL21 *E. coli* expression strain and induced with 0.5mM IPTG

1 for fusion protein expression. The fusion protein was purified using a nickel column.

HIG-82 cells were grown as described above and transfected with a reporter plasmid expressing the luciferase gene driven by the p21 promoter. The HIG-82 cells were transfected with p21-luciferase plasmid by calcium phosphate methods. In addition, as positive controls, a CMV promoter driven p53 plasmid and an Adp53 viral vector were transfected into
 6 HIG-82 cells together with the reporter plasmid expressing the luciferase gene driven by the p21 promoter. Purified pep5/p53/his was added to the culture 6 hours post addition of the reporter plasmid expressing the luciferase gene driven by the p21 promoter.

The reporter plasmid expresses the luciferase gene when p53 binds to the p21 promoter. Therefore, the presence of p53 in cells transfected with the reporter plasmid may be monitored by the presence of luciferase activity in the cells. To check for the presence of luciferase activity in the cells, the cells were washed 2x with PBS, harvested and lysed. The cellular lysate was used in a luciferase activity assay performed using a luciferase assay kit (Promega, Madison, WI).

Figure 20 shows the ability of pep5:p53 to induce p21 promoter driven luciferase
 16 expression in HIG-82 cells. Pep5:p53 was able to induce luciferase expression to similar levels as a plasmid expressing p53 and was much more effective than an adenovirus vector expressing p53. These data indicate that the internalizing peptides of the present invention can effectively deliver and internalize p53 to cells.

Example 8: Facilitation of the Delivery of GST Fusion Proteins to Cells Using Glutathione-
 21 linked Internalizing Peptides of the Present Invention

1 GST-eGFP (glutathione-S-transferase tagged green fluorescent protein), having in
addition a histidine tag, was expressed in E. coli and purified using conventional techniques
using a Nickel column to which the histidine tag binds (*see* Mi et al., *Mol. Ther.* (2000) in press).
The purified GST-eGFP (200 μ l of 0.8 mg/ml/ total of 0.16 mg in TBS containing 1mM CaCl₂
and 10 mM MgCl₂) was incubated together with 50 μ l pep5 (SEQ ID NO:5; 2 mg/ml in TBS) in a
6 total volume of 500 μ l by rotating overnight at 4°C. The mixture was then dialyzed against TBS
at 4°C for 2 hours with one change of buffer.

Hig-82 cells were grown between 80% to 100% confluency in 12 well plates. The
cells were washed 2x with 1 ml of TBS containing 1mM CaCl₂ and 10 mM MgCl₂, and 0.1%
BSA. After the final wash, various dilutions of the glutathione-pep5-GST-eGFP complex (4X,
10X, 20X) were added to the cells, as well as the negative control (GST-eGFP alone) and
“enriched” TBS. The cells were incubated together with the complex or controls at 37°C for 2
hours. The cells were then washed with “enriched” TBS 3x and examined by fluorescent
microscopy.

Figure 21 shows that the glutathione-pep5:GST-eGFP complex was very
16 effectively internalized by HIG-82 cells (panel A) as compared to the GST-eGFP alone (panel B)
indicating that glutathione linked internalizing peptides of the present invention are useful for
facilitating the uptake of GST proteins to target cells.

1 We Claim:

1. A peptide having an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRL LHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRH SK (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPTSTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41), SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43), SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45), SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47), SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),

1 LNVPPSWFLSQR(SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
(SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
AIPNNQLGFPEK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
6 (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR(SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

2. The peptide of claim 1 wherein said peptide facilitates cellular
16 internalization of a cargo linked thereto.

3. The peptide of claim 2 wherein the peptide has the amino acid sequence
KRIHPRLTRSIR (SEQ ID NO:2).

4. The peptide of claim 2 wherein the peptide has the amino acid sequence

1 PPRLRKRRQLNM (SEQ ID NO:3).

5. The peptide of claim 2 wherein the peptide has the amino acid sequence
PIRRRKKLRLK (SEQ ID NO:4).

6 6. The peptide of claim 2 wherein the peptide has the amino acid sequence
RRQRRTSKLMKR(SEQ ID NO:5).

7. The peptide of claim 1 wherein the peptide provides for nuclear
translocation in a target cell.

8. A peptide-cargo complex comprising a peptide and a cargo wherein the
peptide has an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ
ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3),
PIRRRKKLRLK (SEQ ID NO:4), RRQRRTSKLMKR(SEQ ID NO:5), MHKRPTTPSRKM
(SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLHNNLRR
(SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHKS (SEQ ID NO:13),
PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ
(SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
21 HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF

1 (SEQ ID NO:27), LTRNYEAWVPTP(SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29),
 YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPTSTLP (SEQ ID NO:31), AVPAENALNNPF
 (SEQ ID NO:32), SFHQFARATLAS(SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
 HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
 LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD
 6 (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
 SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
 SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
 LNVPPSWFLSQR(SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
 (SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
 AIPNNQLGFPEK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
 (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
 HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
 16 (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLP (SEQ ID NO:64),
 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
 QAASRVENYMHR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
 SNPWDSL SVST (SED ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
 EPDNWSLDFPRR(SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72)
 21 GVVGKLGQRRKKQRRQKK (SEQ ID NO:73),

1 GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

9. The peptide-cargo complex of claim 8 wherein the cargo is selected from
the group consisting of a polynucleotide, a polypeptide, a small molecule, a virus, a modified
6 virus, a viral vector, and a plasmid.

10. The peptide-cargo complex of claim 9 wherein the cargo is a virus
selected from the group consisting of adenovirus, adeno-associated virus, herpes simplex virus,
and retrovirus.

11. The peptide-cargo complex of claim 8 wherein the cargo is selected from
the group consisting of therapeutic proteins, suicide proteins, tumor suppressor proteins,
transcription factors, kinase inhibitors, kinases, cell cycle regulatory proteins, apoptotic proteins,
anti-apoptotic proteins, viral antigens, cellular antigens, differentiation factors, immortalization
16 factors and toxins.

12. The peptide-cargo complex of claim 8 wherein the peptide facilitates
cellular internalization of cargo linked thereto.

21 13. The peptide-cargo complex of claim 12 wherein the peptide provides for

nuclear translocation of said peptide-cargo complex in a target cell.

14. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence KRIHPRLTRSIR (SEQ ID NO:2).

15. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence PPRLRKRRQLNM (SEQ ID NO:3).

16. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence PIRRRKKLRRLK (SEQ ID NO:4).

17. The peptide-cargo complex of claim 8 wherein the peptide has the amino acid sequence RRQRRTSKLMKR (SEQ ID NO:5).

18. The peptide-cargo complex of claim 8 wherein the peptide is biotinylated and the cargo is avidin labeled.

19. The peptide-cargo complex of claim 11, wherein the cargo is an apoptotic protein selected from the group consisting of p53, caspase-3, HSV thymidine kinase and an antimicrobial peptide.

1

20. The peptide-cargo complex of claim 19, wherein the antimicrobial peptide is selected from the group consisting of KLAKLAK (SEQ ID NO:22) and KLAKLAKKLAKLAK (SEQ ID NO:23).

6

21. The peptide-cargo complex of claim 19 wherein having an amino acid sequence RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24).

22. The peptide-cargo complex of claim 8 wherein the cargo is glutathione.

23. A method for identifying peptides capable of cellular internalization of cargo linked thereto, said method comprising:

incubating a target cell with a peptide display library;

isolating internalized peptides presented by said peptide display library from said target cells and identifying said peptides;

16

synthesizing said peptides;

linking said peptides to cargo to form a peptide-cargo complex;

incubating said peptide-cargo complex with a target cell; and

determining the ability of said peptide to facilitate the cellular internalization of said cargo into said target cell.

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24. The method of claim 23 further comprising determining the ability of said peptide to facilitate the nuclear localization of said cargo.

25. The method of claim 23 wherein said peptide display library is an M13 phage display library.

26. The method of claim 25 wherein said phage display library comprises peptides which comprise 12 amino acids.

27. The method of claim 23 further comprising biotin labeling the peptides during or after said synthesis of the peptides.

28. The method of claim 23 further comprising avidin labeling the cargo.

29. The method of claim 23 wherein the cargo is selected from the group consisting of a polynucleotide, a polypeptide, a small molecule, a virus, a modified virus, a viral vector, and a plasmid.

30. An expression cassette comprising a DNA encoding a fusion protein comprising a leader sequence, a protein of interest and an internalizing peptide having an amino acid sequence selected from the group consisting of KRRIQRILSRNS (SEQ ID NO:1),

1 KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKCLRRLK
(SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI
(SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLHNNLRR (SEQ ID NO:11),
HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHKS (SEQ ID NO:13), PPHNRIQRRLNM
6 (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA
(SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),
LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT
(SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH
(SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),
FQPYDHPAEVSYS (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),
FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
16 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
(SEQ ID NO:52), VSKQPYYMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
21 AIPNNQLGFPEK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT

(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
 HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
 (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIIITSPYLP (SEQ ID NO:64),
 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
 QAASRVENYMHR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
 SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
 EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
 GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
 GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
 GRRTKKQRRQKKPP (SEQ ID NO:75).

31. The expression cassette of claim 30 further comprising expression control
 sequences operatively linked to said DNA.

32. A transfer vector comprising the expression cassette of claim 31.

33. The expression cassette of claim 30, wherein said leader sequences are
 selected from the group consisting of IL-1ra, PTH, VP-22 and related sequences.

34. The expression cassette of claim 30 wherein the protein of interest is
 selected from the group consisting of apoptotic protein, anti-apoptotic protein, cell cycle

1 regulatory protein, transcription factor, suicide gene product, viral or tumor antigens, and cell
proliferation factors..

35. The expression cassette of claim 30, wherein the encoded fusion protein
comprises an amino acid sequence which facilitates removal of leader sequences therefrom and
6 wherein said leaderless fusion protein comprises an internalizing peptide and a protein of
interest.

36. The expression cassette of claim 30 wherein said fusion protein encoded
thereby is produced and secreted from a cell and subsequently internalized into surrounding cells.

37. A method for inducing synovial cell death comprising administering a
peptide-cargo complex to said synovial cell.

38. A method for inducing apoptosis in a tumor cell comprising administering
16 a peptide-cargo complex to said tumor cell.

39. A method for reducing white blood cells in arthritic joints comprising
administering a peptide-cargo complex to said white blood cells.

40. The method of claim 37, 38 or 39 wherein the peptide has an amino acid

1 sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1),
 KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK
 (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),
 RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI
 (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRL LHNNLRR (SEQ ID NO:11),
 6 HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRH SK (SEQ ID NO:13), PPHNRIQRRLNM
 (SEQ ID NO:14), SMLKR NHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),
 SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA
 (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),
 LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT
 (SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),
 SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH
 (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),
 FQPYDHPAEVS Y (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),
 FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
 16 SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
 SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
 LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
 21 (SEQ ID NO:52), VSKQPYYMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),

1 AIPNNQLGFPFK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
(SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
6 QAASRVENYMHR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

41. The method of claim 37, 38 or 39 wherein the cargo is an apoptotic
protein.

42. The method of claim 37, 38 or 39 wherein the apoptotic protein is selected
from the group consisting of p53, caspase-3, HSV thymidine kinase and an antimicrobial
peptide.

43. The method of claim 41 wherein the antimicrobial peptide has an amino

acid sequence selected from the group consisting of KLAKLAK (SEQ ID NO:22) and KLAKLAKKLAKLAK (SEQ ID NO:23).

44. The method of claim 37, 38 or 39 wherein the peptide-cargo complex has an amino acid sequence RRQRRTSKLMKRGGKLAKLAKKLAKLAK (SEQ ID NO:24).

45. A method for internalizing a GST-fusion protein into a cell comprising administering to said cell a peptide-cargo complex and a GST fusion protein.

46. The method of claim 45 wherein the peptide has an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6), RQSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRL LHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRH SK (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPTSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),

1 SFHQFARATLAS(SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH
(SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),
FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),
FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
6 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
LNVPPSWFLSQR(SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
(SEQ ID NO:52), VSKQPYYMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
AIPNNQLGFPPK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
(SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
16 QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR(SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
21 GRRTKKQRRQKKPP (SEQ ID NO:75).

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47. The method of claim 45 wherein the cargo is glutathione.

48. A kit for internalizing a GST-fusion protein into a cell comprising a peptide-cargo complex.

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49. The kit according to claim 48 wherein the peptide has the amino acid

sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID NO:1),

KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK

(SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6),

RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI

(SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLHNNLRR (SEQ ID NO:11),

HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHRSK (SEQ ID NO:13), PPHNRIQRRLNM

(SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16),

SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA

(SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27),

16

LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT

(SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32),

SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH

(SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36), LTMPSDLQPVLW (SEQ ID NO:37),

FQPYDHPAEVSYS (SEQ ID NO:38), FDPFFWKYSPRD (SEQ ID NO:39),

21

FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),

1 SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
 SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
 LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
 6 (SEQ ID NO:52), VSKQPYMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
 AIPNNQLGFPFK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
 (SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPALLTVH (SEQ ID NO:59),
 HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
 (SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLPS (SEQ ID NO:64),
 TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
 QAASRVENYMHHR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
 SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
 EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
 GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
 16 GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
 GRRTKKQRRQKKPP (SEQ ID NO:75).

50. The kit according to claim 48 wherein the cargo is glutathione.

21 51. An immunogen comprising a peptide-cargo complex wherein said peptide

1 has an amino acid sequence selected from the group consisting of KRIIQRILSRNS (SEQ ID
 NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3),
 PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM
 (SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8),
 SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRLLHNNLRR
 6 (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHSHK (SEQ ID NO:13),
 PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ
 (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18),
 HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF
 (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29),
 YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPSTTLP (SEQ ID NO:31), AVPAENALNNPF
 (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34),
 HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),
 LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHAEVSY (SEQ ID NO:38), FDPFFWKYSPRD
 (SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
 16 SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
 SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
 SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
 LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
 21 (SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),

1 AIPNNQLGFPFK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPLALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
(SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIITSPYLP (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
6 QAASRVENYMH (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
EPDNWSLDFPRR (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75).

52. The immunogen of claim 51 wherein the peptide has an amino acid
sequence RRQRRTSKLMKR (SEQ ID NO:5).

16 53. The immunogen of claim 51 wherein the peptide has an amino acid
sequence GVVGKLGQRRTKKQRRQKK (SEQ ID NO:73).

21 54. The immunogen of claim 51 wherein the cargo is selected from the group
consisting of a polynucleotide, a polypeptide, a protein, a virus, a modified virus, a viral vector,
and a plasmid.

1

55. The immunogen of claim 51 wherein the cargo is an antigen.

56. The immunogen of claim 51 wherein the cargo is an HIV protein selected from the group consisting of Gag, Pol, Env, Tat, Nef, Vpr, Vpv, Rev.

6

57. A method for eliciting an immune response in a subject comprising administering to a target cell of said subject an immunogen comprising a peptide-cargo complex wherein said peptide has an amino acid sequence selected from the group consisting of KRIQRILSRNS (SEQ ID NO:1), KRIHPRLTRSIR (SEQ ID NO:2), PPRLRKRRQLNM (SEQ ID NO:3), PIRRRKKLRRLK (SEQ ID NO:4), RRQRRTSKLMKR (SEQ ID NO:5), MHKRPTTPSRKM (SEQ ID NO:6), RQRSRRRPLNIR (SEQ ID NO:7), RIRMIQNLIKKT (SEQ ID NO:8), SRRKRQRSNMRI (SEQ ID NO:9), QRIRKSKISRTL (SEQ ID NO:10), PSKRL LHNNLRR (SEQ ID NO:11), HRHIRRQSLIML (SEQ ID NO:12), PQNRLQIRRHISK (SEQ ID NO:13), PPHNRIQRRLNM (SEQ ID NO:14), SMLKRNHSTSNR (SEQ ID NO:15), GSRHPSLIIPRQ (SEQ ID NO:16), SPMQKTMNLPPM (SEQ ID NO:17), NKRILIRIMTRP (SEQ ID NO:18), HGWZIHGLLHRA (SEQ ID NO:25), AVPAKKRZKSV (SEQ ID NO:26), PNTRVRPDVSF (SEQ ID NO:27), LTRNYEAWVPTP (SEQ ID NO:28), SAETVESCLAKSH (SEQ ID NO:29), YSHIATLPFTPT (SEQ ID NO:30), SYIQRTPTSTLP (SEQ ID NO:31), AVPAENALNNPF (SEQ ID NO:32), SFHQFARATLAS (SEQ ID NO:33), QSPTDFTFPNPL (SEQ ID NO:34), HFAAWGGWSLVH (SEQ ID NO:35), HIQLSPFSQSWR (SEQ ID NO:36),

16

21

1 LTMPSDLQPVLW (SEQ ID NO:37), FQPYDHPAEVSY (SEQ ID NO:38), FDPFFWKYSPRD
(SEQ ID NO:39), FAPWDTASFMLG (SEQ ID NO:40), FTYKNFFWLPEL (SEQ ID NO:41),
SATGAPWKMWVR (SEQ ID NO:42), SLGWMLPFSPPF (SEQ ID NO:43),
SHAFTWPTYLQL (SEQ ID NO:44), SHNWLPLWPLRP (SEQ ID NO:45),
SWLPYPWHVPSS (SEQ ID NO:46), SWWTPWHVHSES (SEQ ID NO:47),
6 SWAQHLSLPPVL (SEQ ID NO:48), SSSIFPPWLSFF (SEQ ID NO:49),
LNVPPSWFLSQR (SEQ ID NO:50), LDITPFLSLTLP (SEQ ID NO:51), LPHPVLHMGPLR
(SEQ ID NO:52), VSKQPYWMWNGN (SEQ ID NO:53), NYTTYKSHFQDR (SEQ ID NO:54),
AIPNNQLGFPPK (SEQ ID NO:55), NIENSTLATPLS (SEQ ID NO:56), YPYDANHTRSPT
(SEQ ID NO:57), DPATNPGPHFPR (SEQ ID NO:58), TLPSPALLTVH (SEQ ID NO:59),
HPGSPFPPEHRP (SEQ ID NO:60), TSHTDAPPARSP (SEQ ID NO:61), MTPSSLSTLPWP
(SEQ ID NO:62), VLGQSGYLMPMR (SEQ ID NO:63), QPIIITSPYLPS (SEQ ID NO:64),
TPKTMTQTYDFS (SEQ ID NO:65), NSGTMQSASRAT (SEQ ID NO:66),
QAASRVENYMR (SEQ ID NO:67), HQHKPPPLTNNW (SEQ ID NO:68),
SNPWDSL SVST (SEQ ID NO:69), KTIEAHPPYYAS (SEQ ID NO:70),
16 EPDNLWLDLFP (SEQ ID NO:71), HQHKPPPLTNNW (SEQ ID NO:72),
GVVGKLGQRRKKQRRQKK (SEQ ID NO:73),
GRRTKKQRRQKKPPRYMILGLLALAAVCSAA (SEQ ID NO:74) and
GRRTKKQRRQKKPP (SEQ ID NO:75)..

21 58. The method of claim 57 wherein the target cell is a mucosal cell.

ABSTRACT

1 The present invention relates to internalizing peptides which facilitate the uptake
and transport of cargo into the cytoplasm and nuclei of cells as well as methods for the
identification of such peptides. The internalizing peptides of the present invention are selected
for their ability to efficiently internalize cargo into a wide variety of cell types both *in vivo* and *in*
6 *vitro*. The method for identification of the internalizing peptides of the present invention
comprises incubating a target cell with a peptide display library, isolating peptides with
internalization characteristics and determining the ability of said peptide to internalize cargo into
a cell.

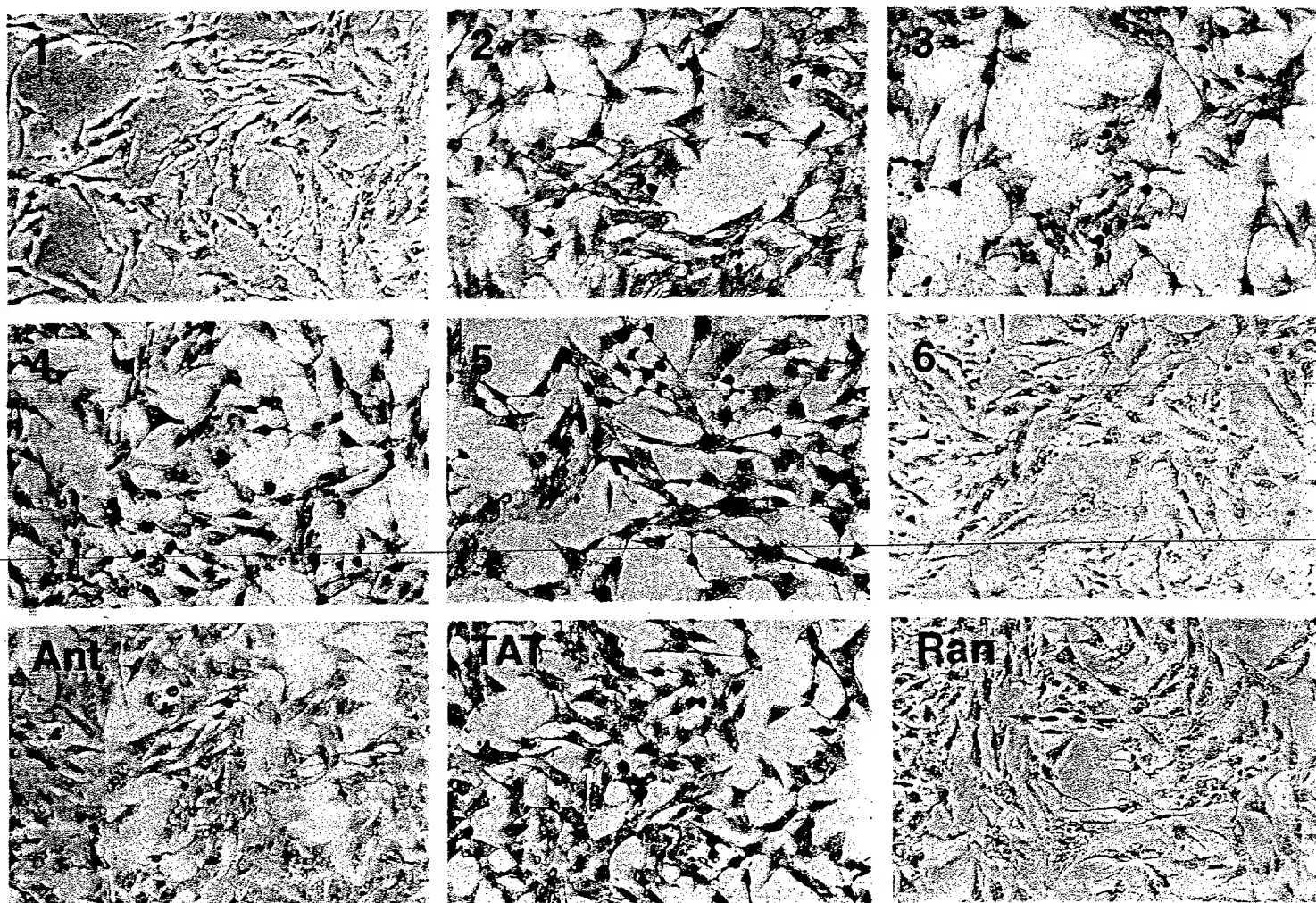


Figure 1A

00653182-083100

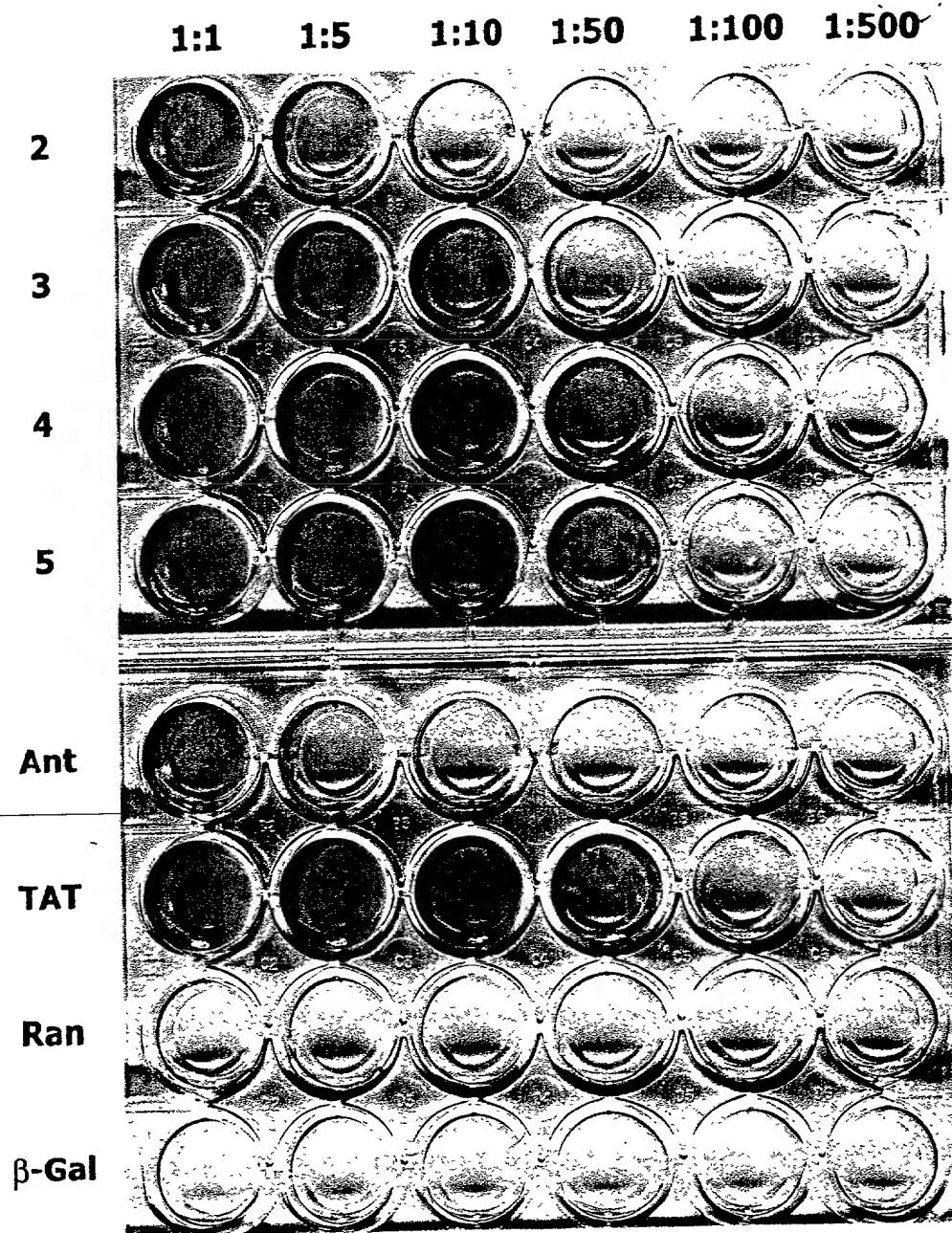


Figure 1B

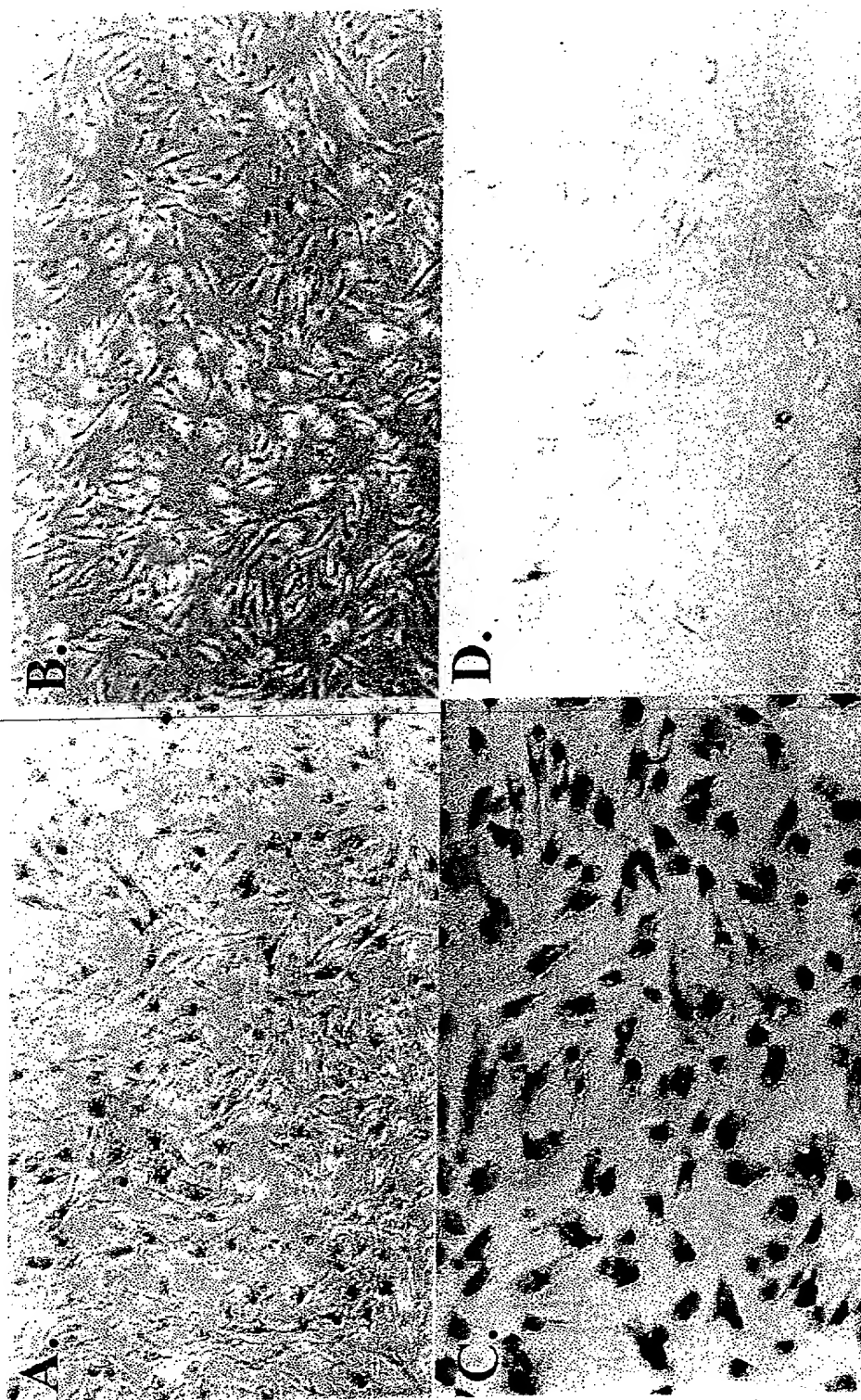


Figure 2

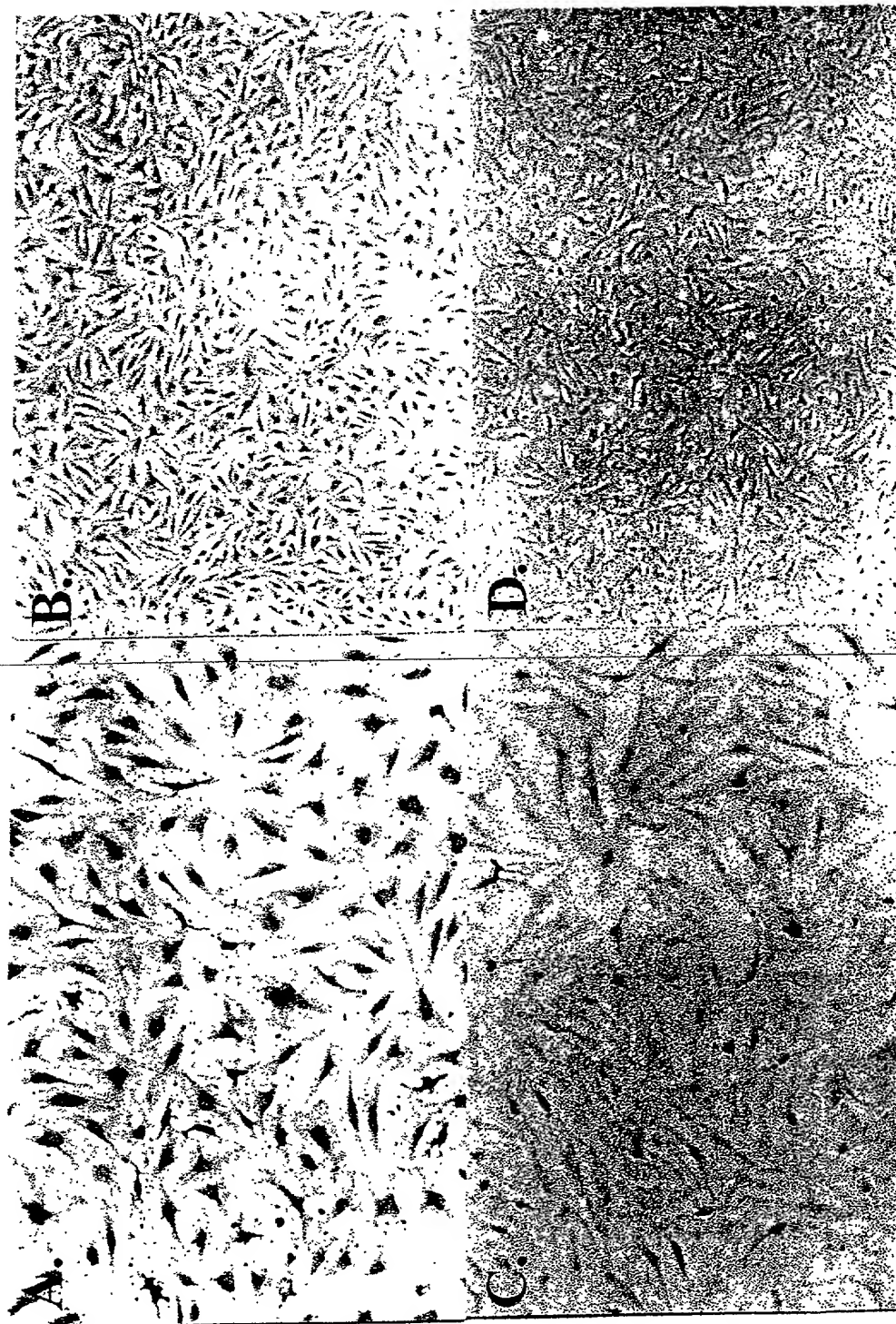


Figure 3

007E80" 28TE5960

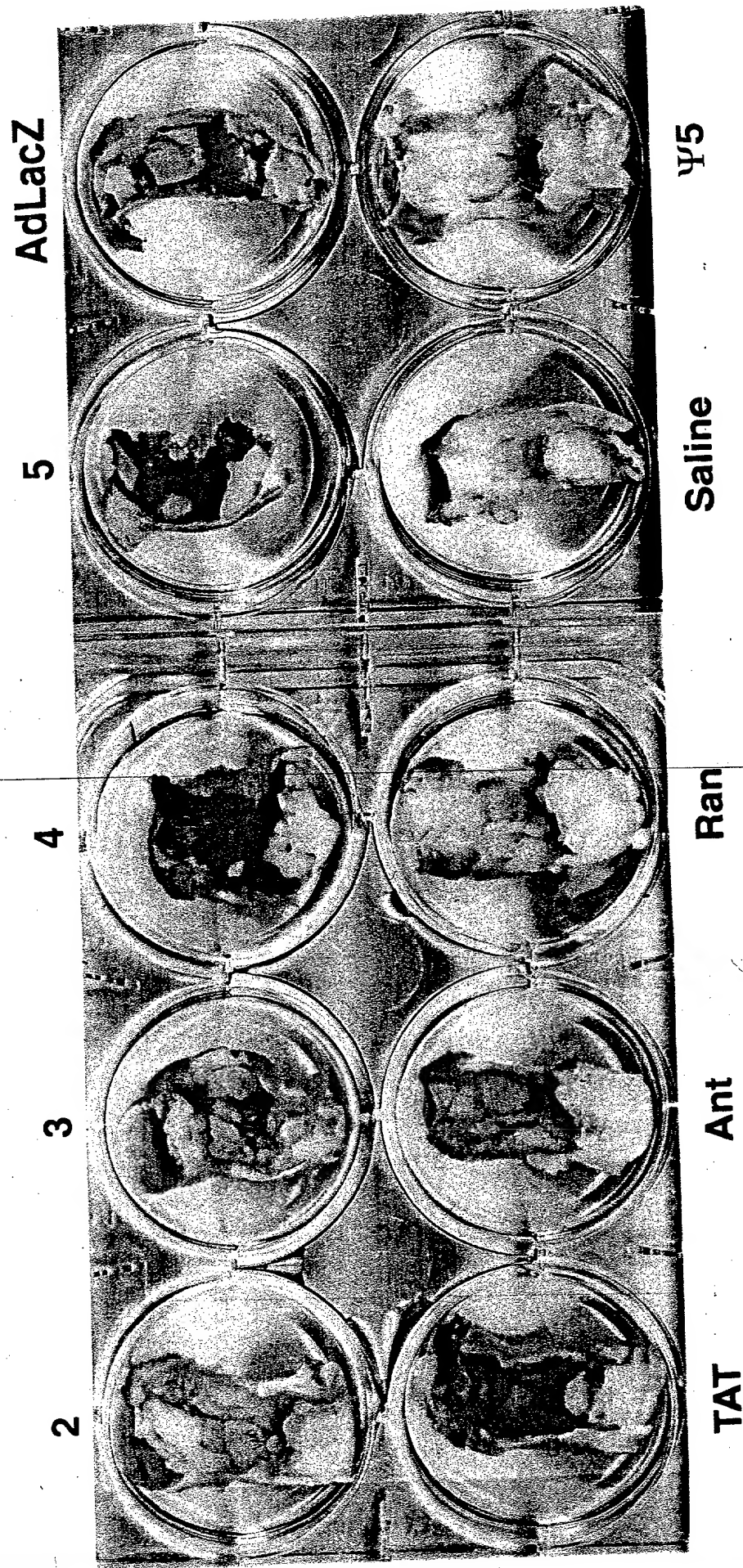


Figure 4A

00T E30" 28T E5950

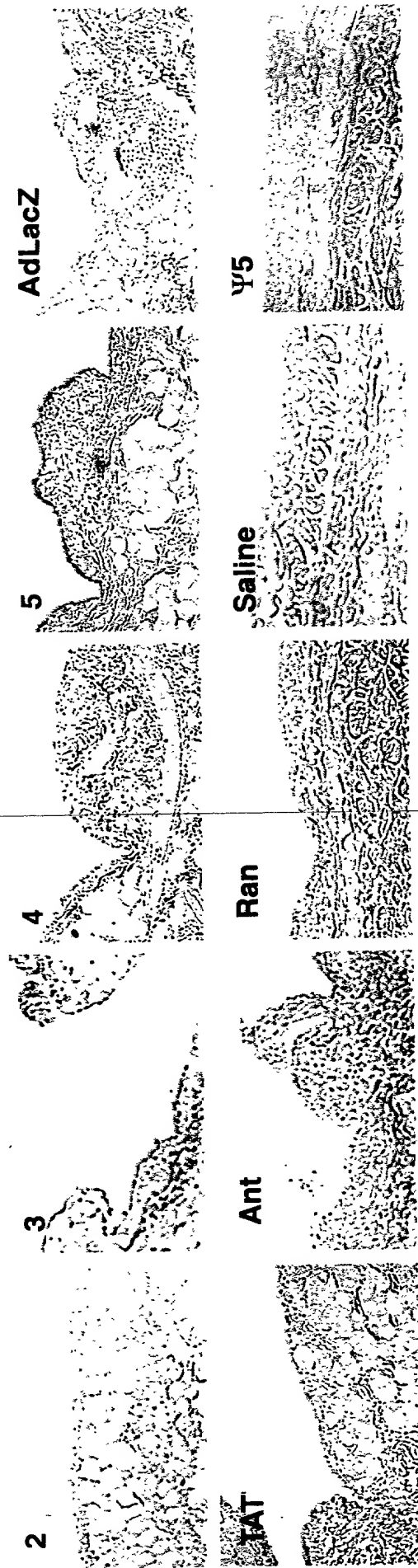


Figure 4B

00F80" 28T55960

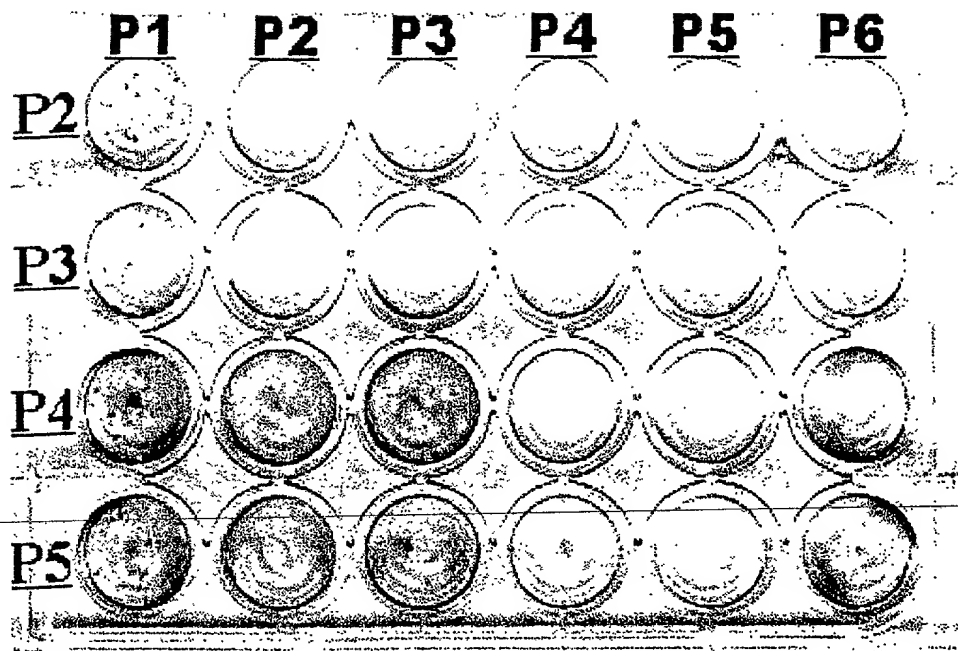


Figure 5

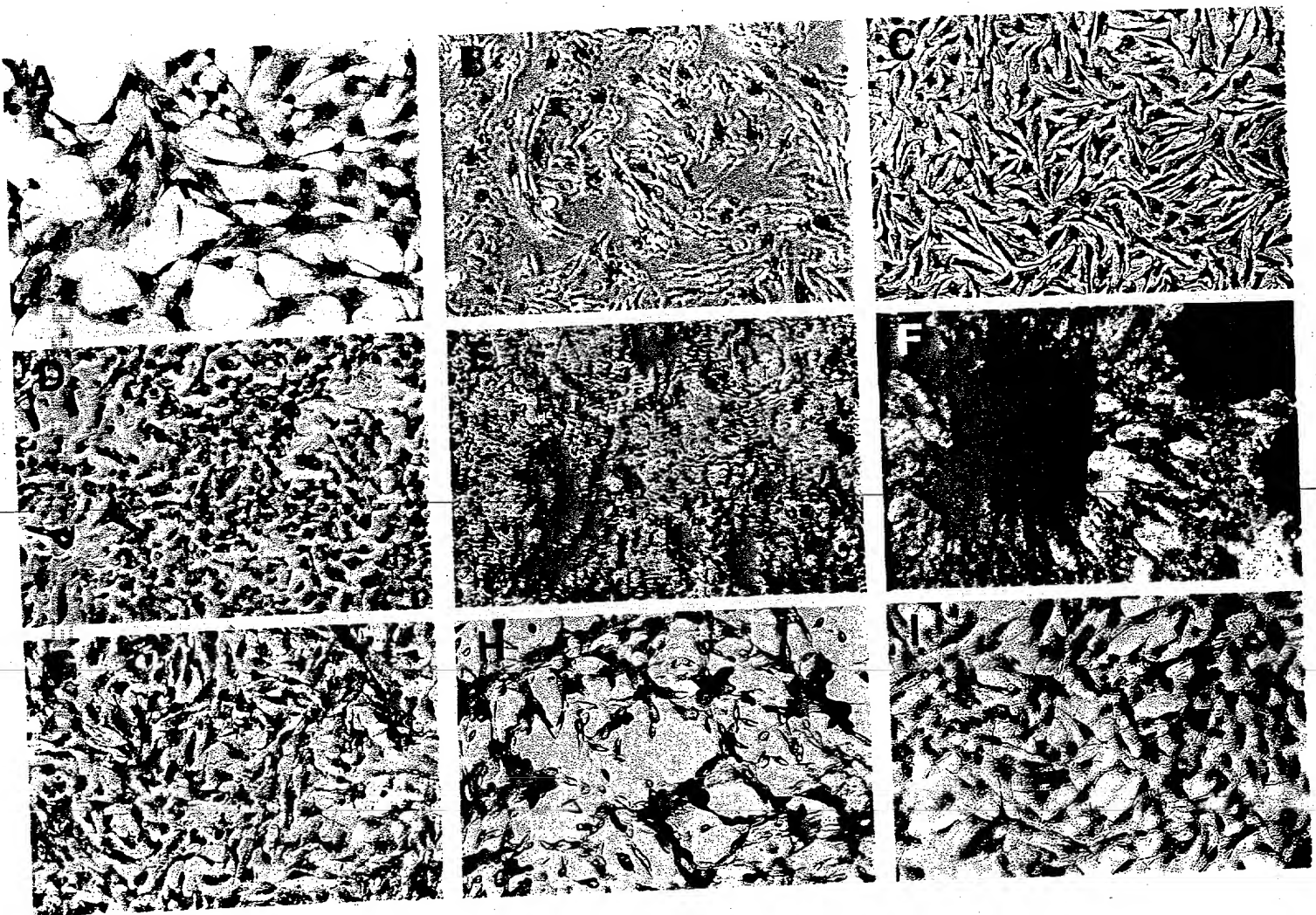


Figure 6

007680" 2875590

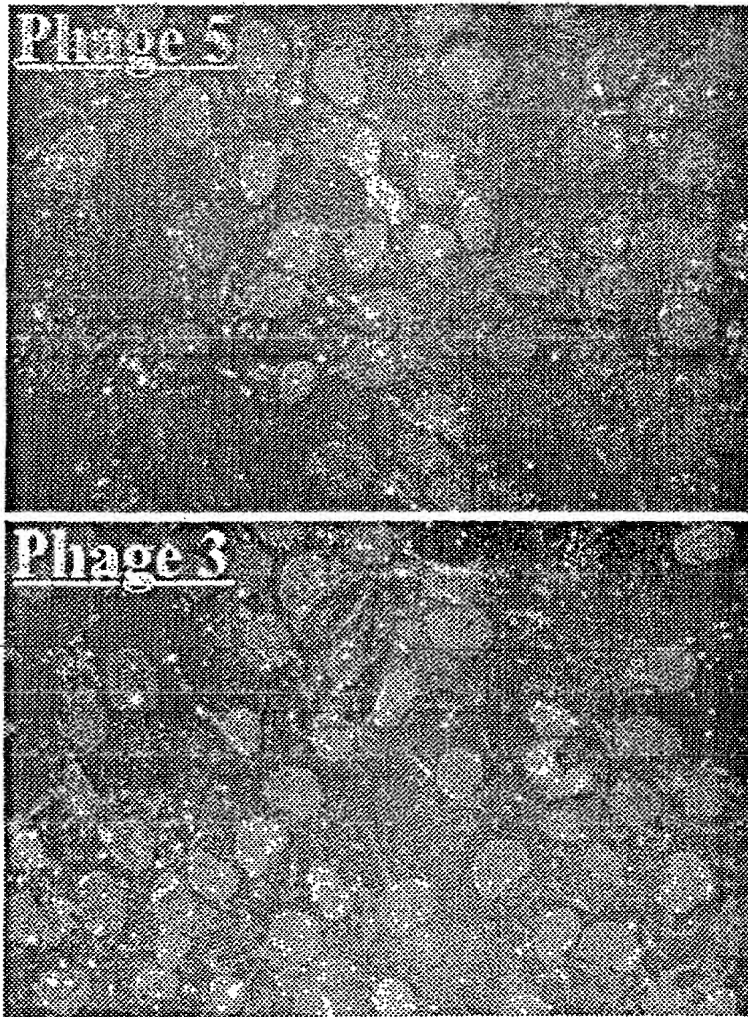


Figure 7

007E80" 28TE5960

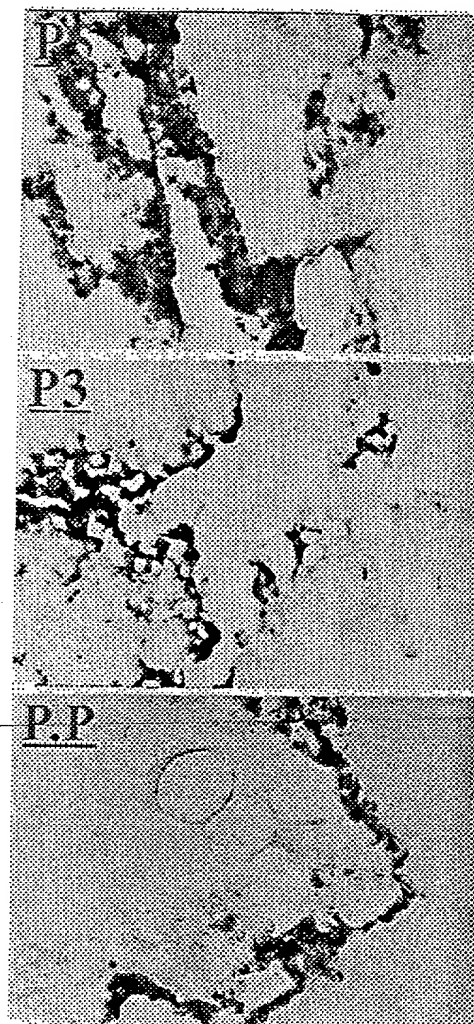


Figure 8

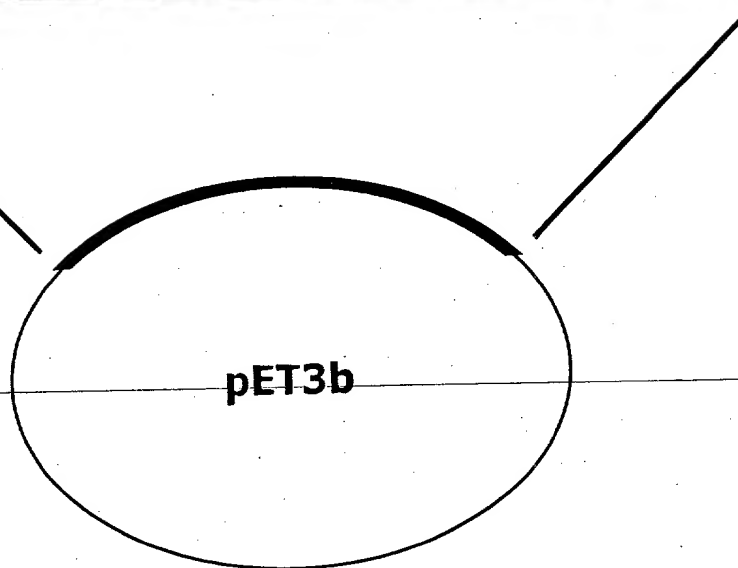
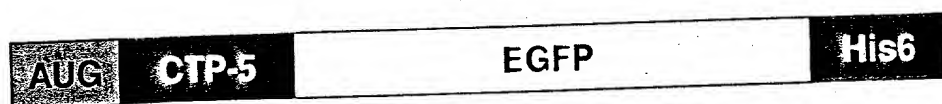


Figure 9A

007E80" 28TE560

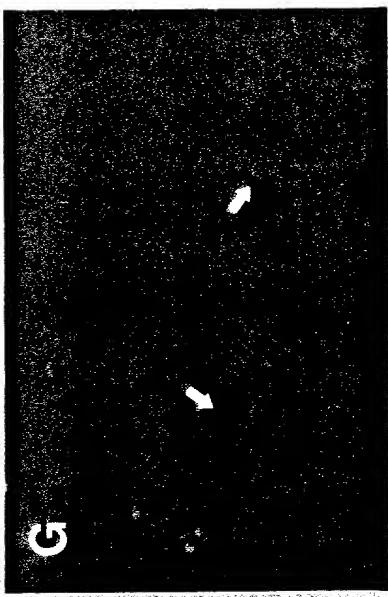
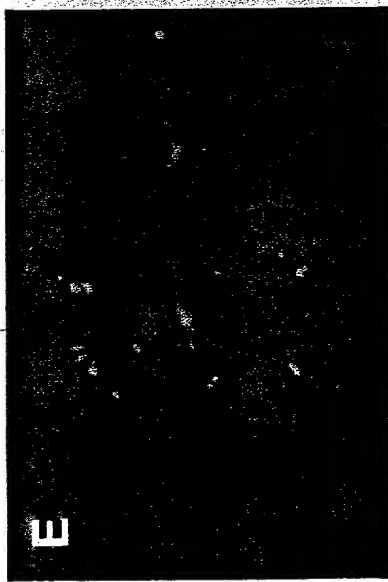
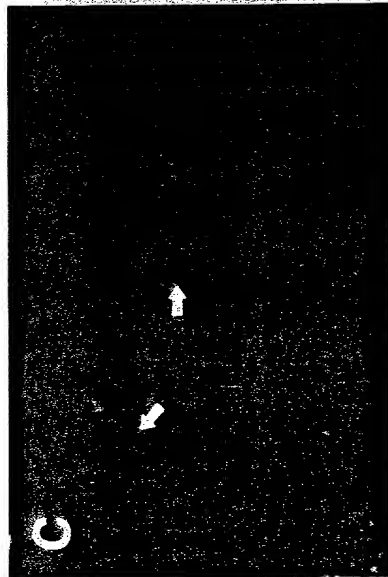
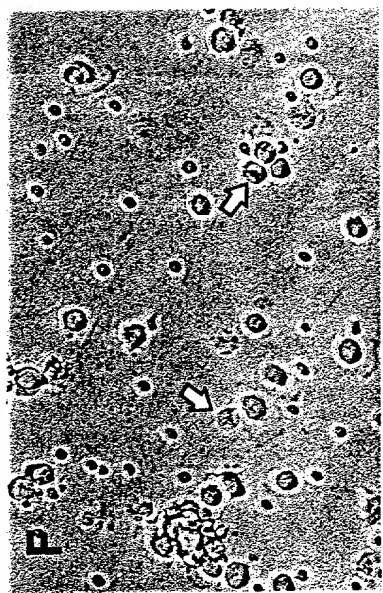
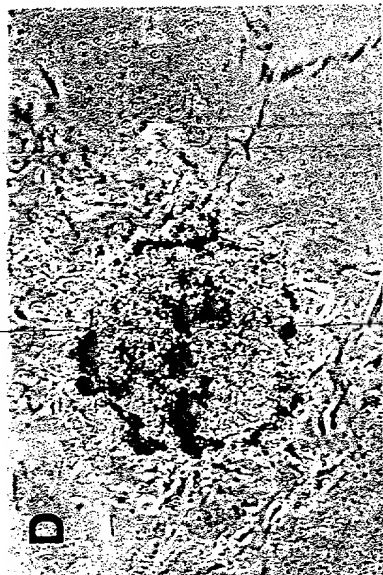
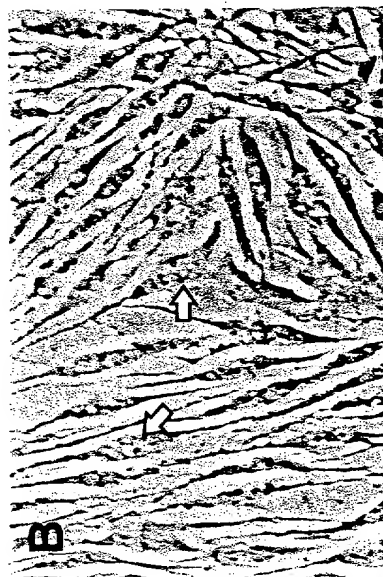


Figure 9B-G



Experiment Type
Wavelength

CD - PMT
Signal: -0.36 m deg
Dynode: 178.17 v
PMT DC: 1.071 v

Fluorescence PMT
Signal: 0.00 Rel Int
Dynode: 0.29 v

Monochromator
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 0.329 mm

Sample
24.98 deg C

RUN EXPERIMENT

Experiment is IDLE

Ready

Data Collection Display

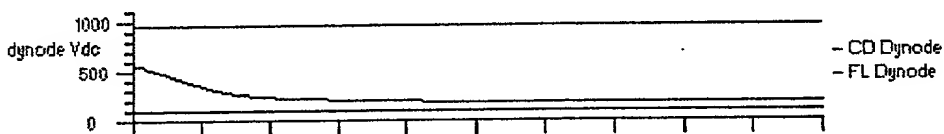
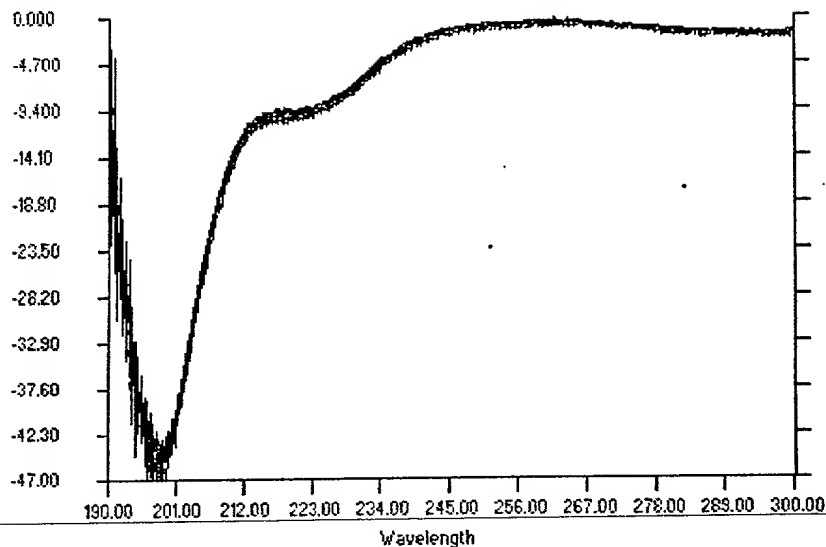


Figure 10 A



Experiment Type
Wavelength

CD - PMT
Signal: -0.73 m deg
Dynode: 178.54 v
PMT DC: 1.071 v

Fluorescence PMT
Signal: -0.00 Rel Int
Dynode: 0.27 v

Monochromator
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 0.331 mm

Sample
24.99 deg C

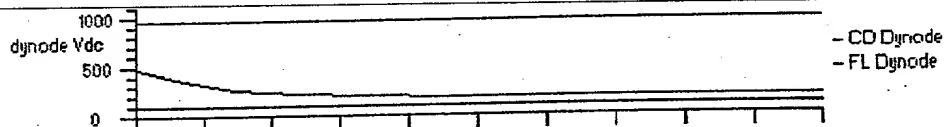
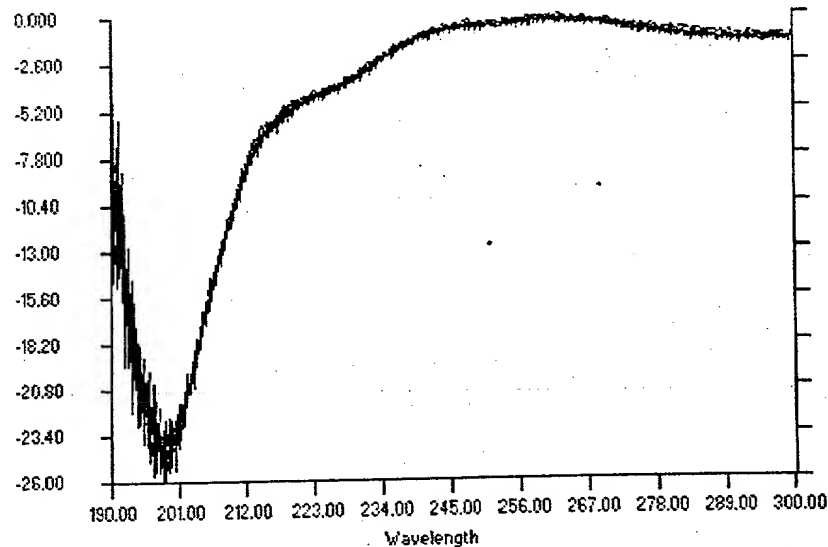
RUN EXPERIMENT

Experiment is IDLE

Ready

217.265, -24.078/85.217

Data Collection Display



005181500

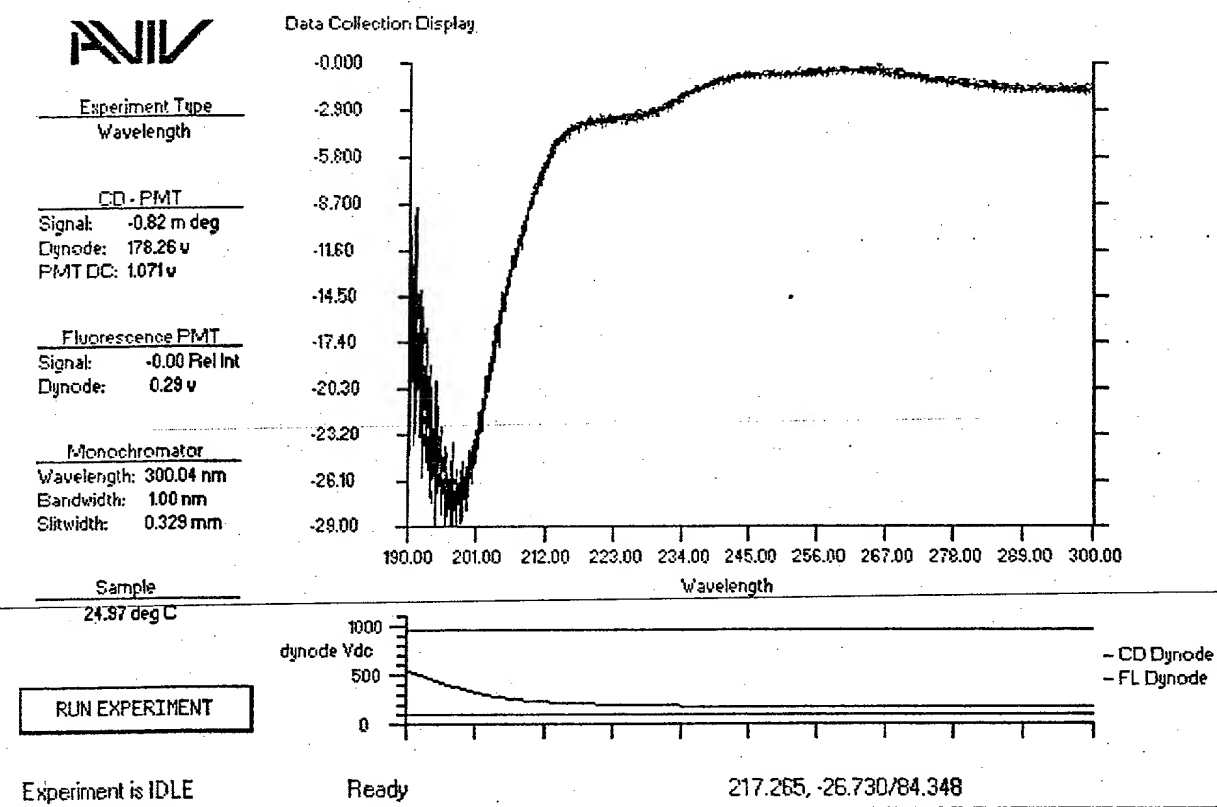


Figure 10 C



Experiment Type
Wavelength

CD - PMT
Signal: -0.76 m deg
Dynode: 179.39 v
PMT DC: 1.071 v

Fluorescence PMT
Signal: -0.03 Rel Int
Dynode: 0.11 v

Monochromator
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 0.331 mm

Sample
24.99 deg C

RUN EXPERIMENT

Experiment is IDLE

Ready

213.818, -35.357/86.087

Data Collection Display

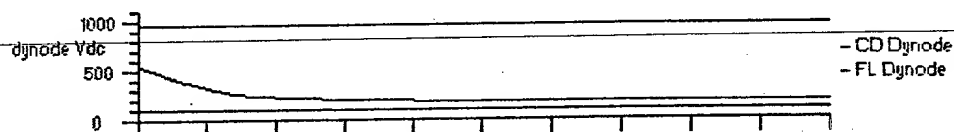
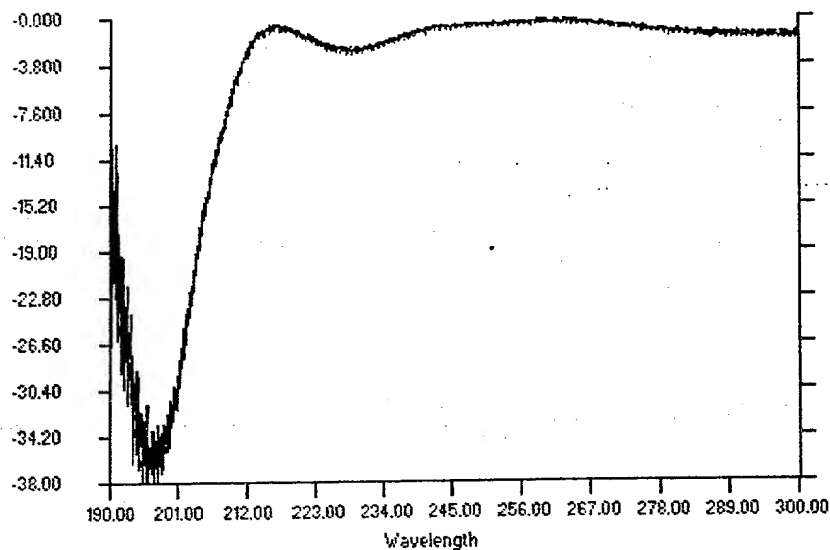


Figure 10 D



Experiment Type
Wavelength

CD - PMT
Signal: -0.99 m deg
Dynode: 177.73 v
PMT DC: 1071 v

Fluorescence PMT
Signal: -0.03 Rel Int
Dynode: 0.46 v

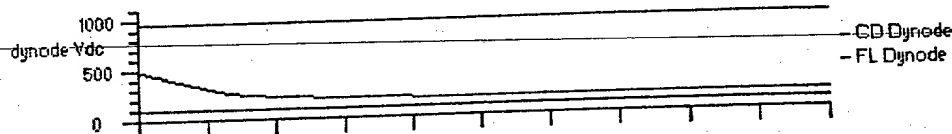
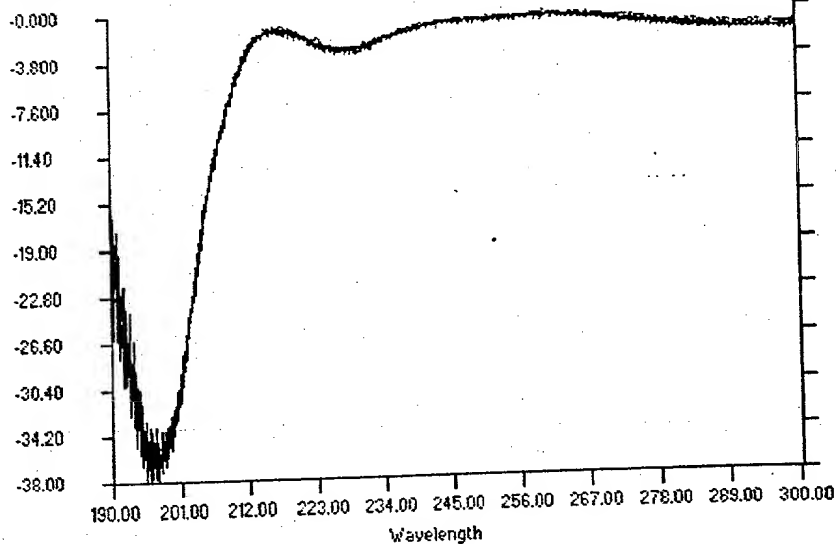
Monochromator
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 0.331 mm

Sample
24.99 deg C

RUN EXPERIMENT

Experiment is IDLE

Data Collection Display



214.758, -34.696/82.609

Ready

Figure 10 E



Experiment Type
Wavelength

CD - PMT
Signal: -0.81 m deg
Dynode: 179.81 v
PMT DC: 1071 v

Fluorescence PMT
Signal: -0.00 Rel Int
Dynode: 0.30 v

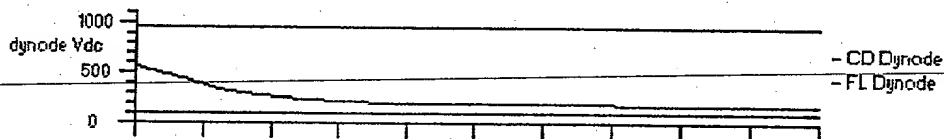
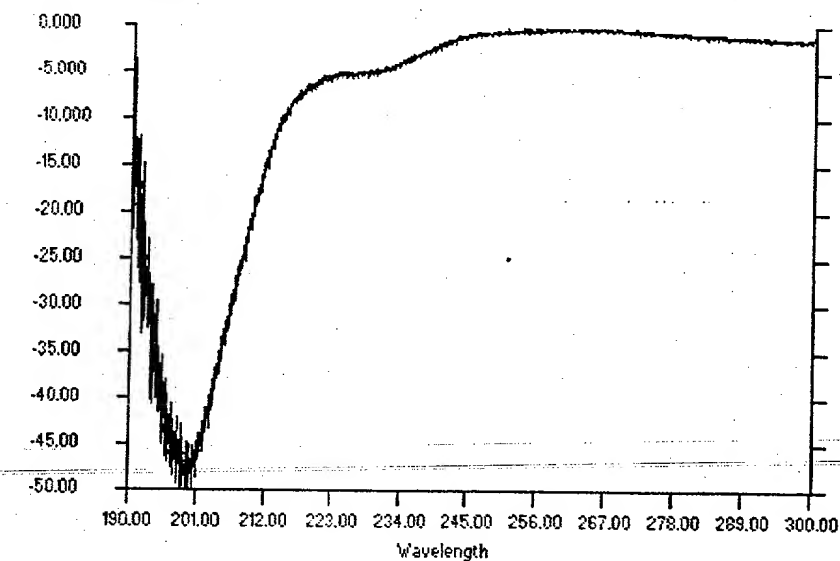
Monochromator
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 0.331 mm

Sample
24.99 deg C

RUN EXPERIMENT

Experiment is IDLE

Data Collection Display



Ready

217.892, -44.130/76.522

Figure 10 F

005131-03100

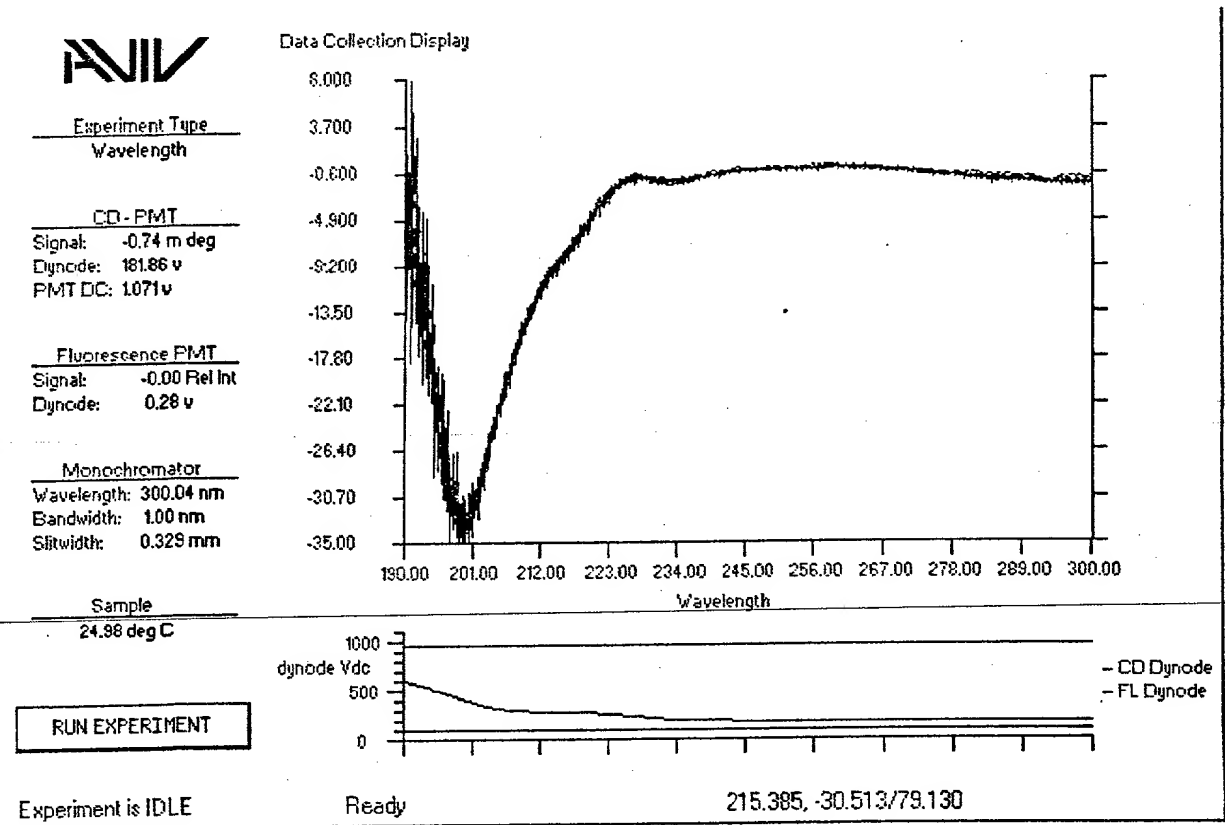


Figure 10 G



Experiment Type
Wavelength

CD-PMT

Signal: -17.65 m deg
Dynode: 530.95 v
PMT DC: 1061 v

Fluorescence PMT

Signal: -0.00 Rel Int
Dynode: 0.30 v

Monochromator

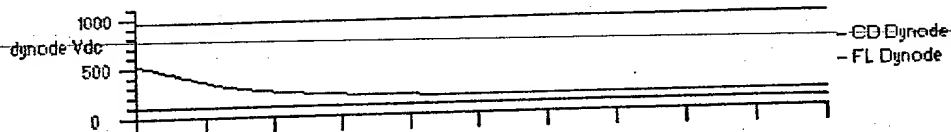
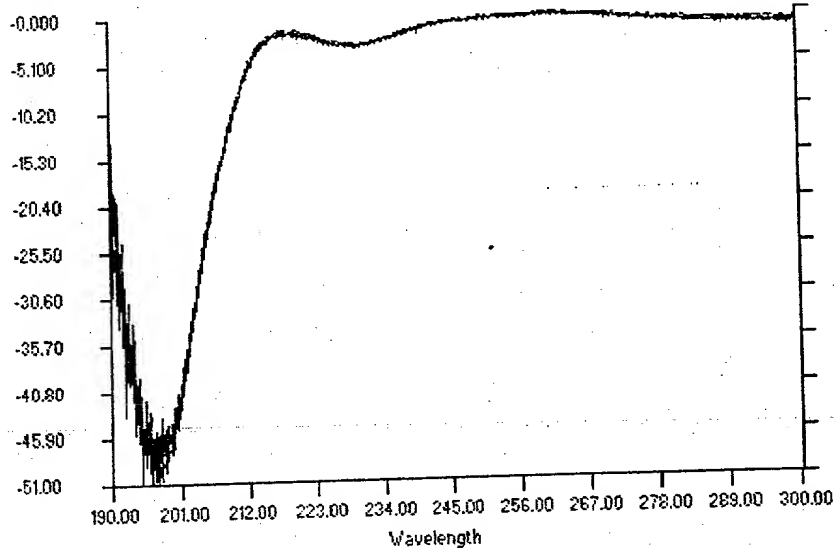
Wavelength: 300.04 nm
Bandwidth: 1.00 nm
Slitwidth: 1.314 mm

Sample

24.99 deg C

STOP EXPERIMENT

Data Collection Display



Ready

Moving slits, please wait...

217.578, -44.791/75.652

Figure 10 H

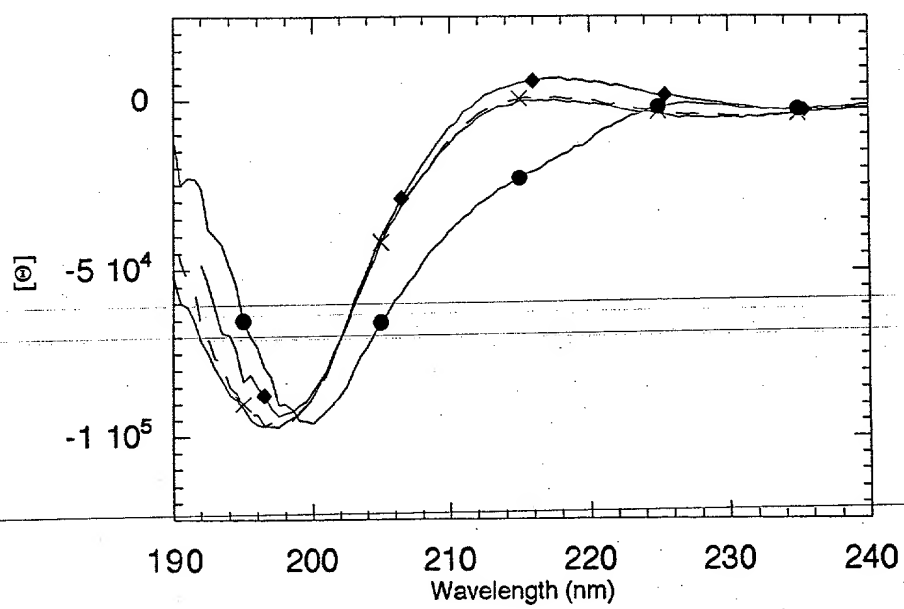


Figure 11A

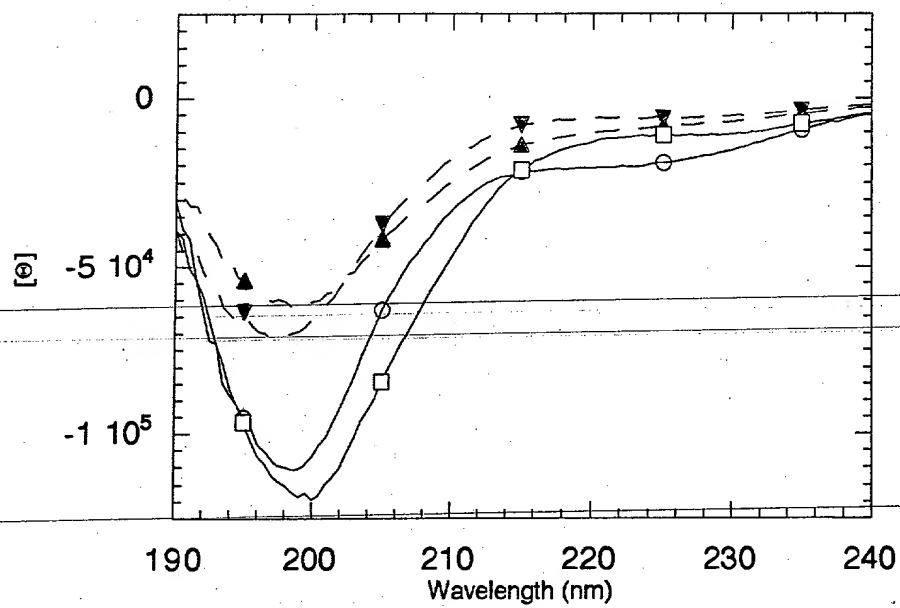


Figure 11B

Non-Biotinylated 100x Excess

Saline Ran Lys 4 5 TAT Ant

Biotinylated

**3
4
5
TAT
Ant
Ran**

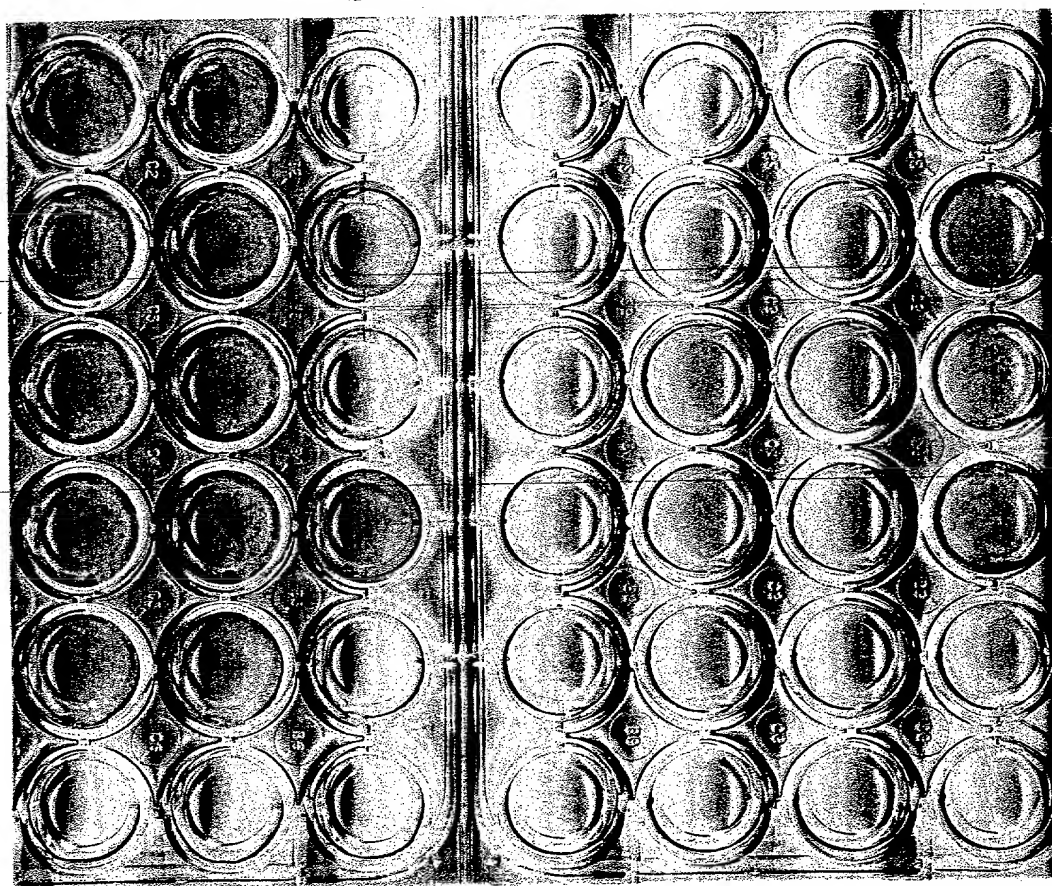


Figure 12

00T680" 22T6500

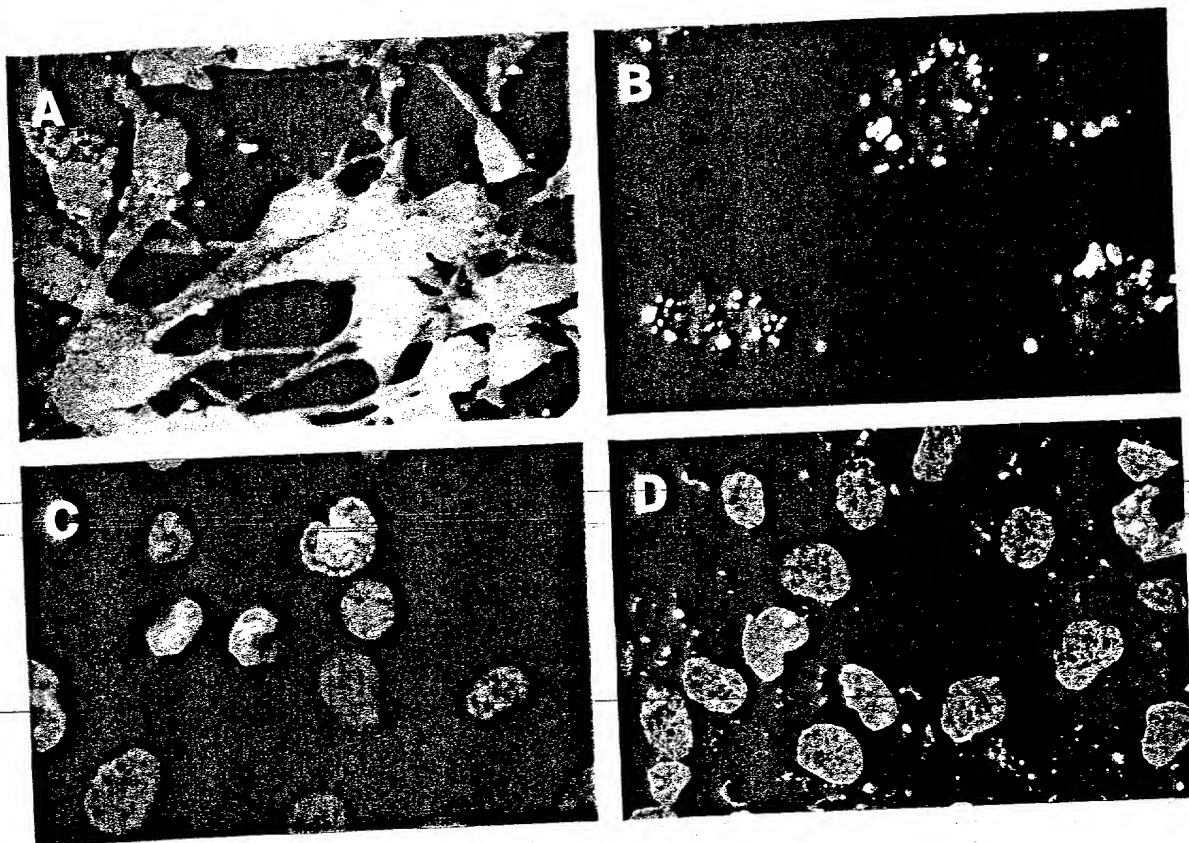


Figure 13

CTP-5-(KLAKLAK)₂ Peptide Impairs Cell Viability in Hig 82 Cells

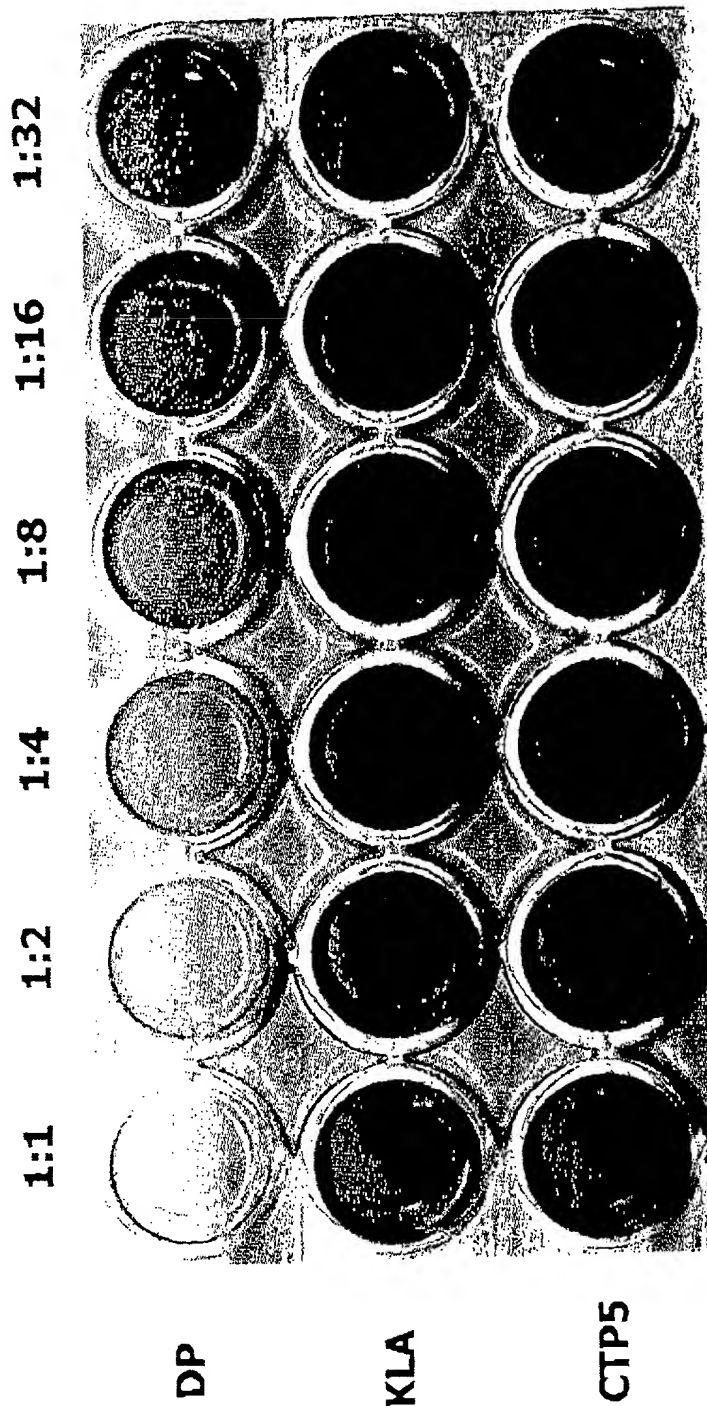


Figure 14

CTP-5-(KLAKLAK)₂ Peptide Impairs Cell Viability in Hlg 82 Cells

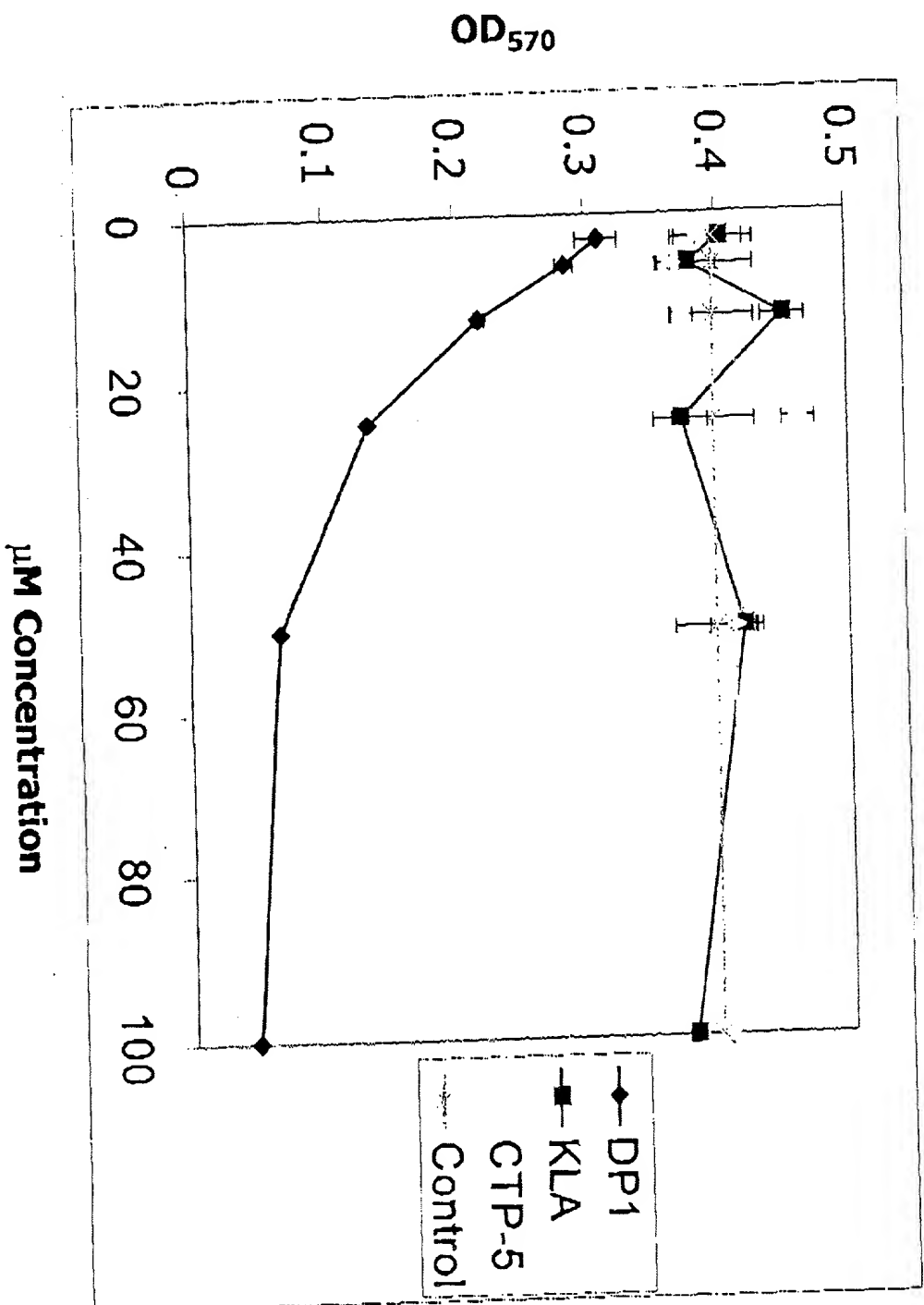


Figure 15

Effect of CTP-5-(KLAKLAK)₂ Peptide Administration on Day 7 MCA205 Tumors

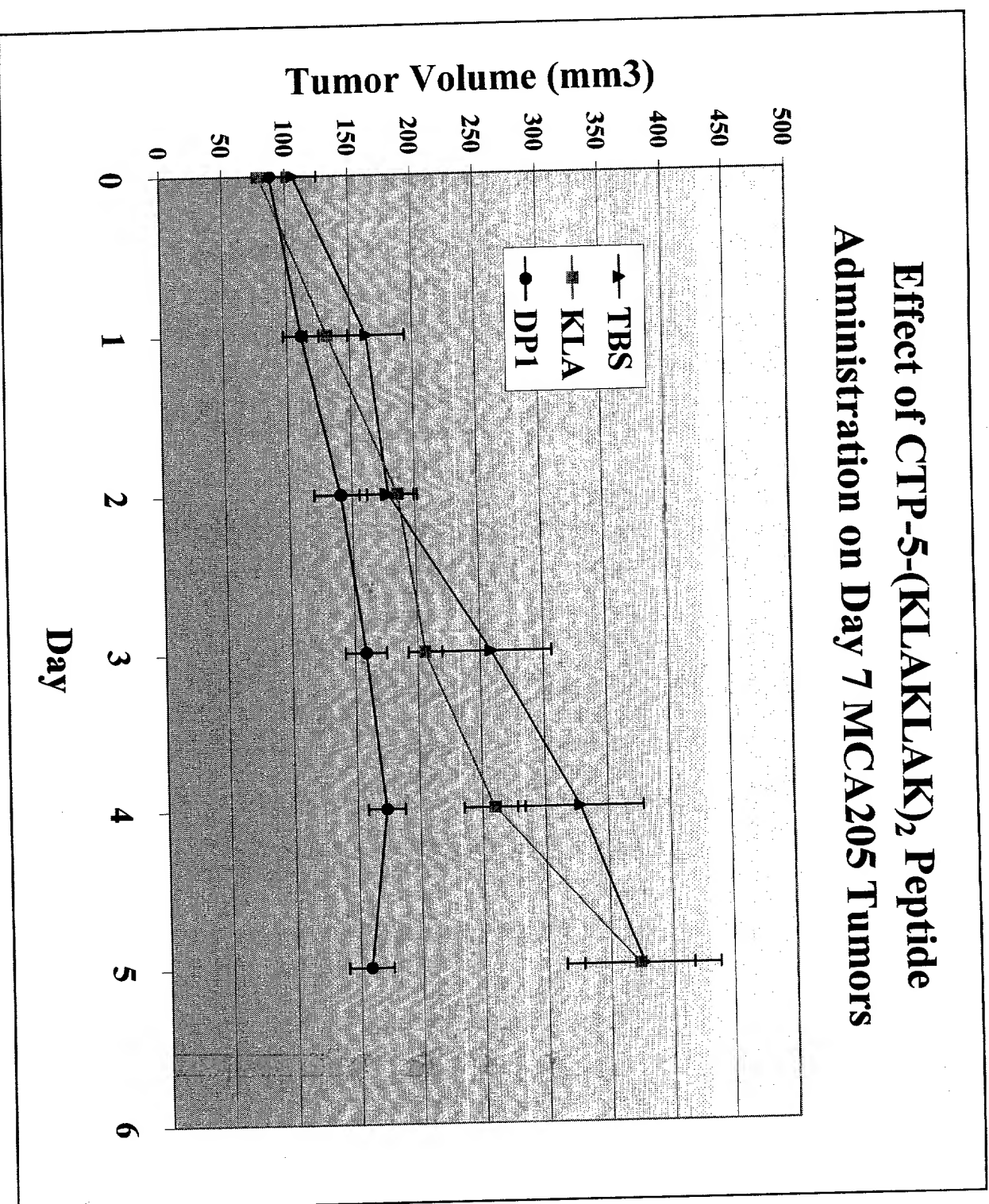
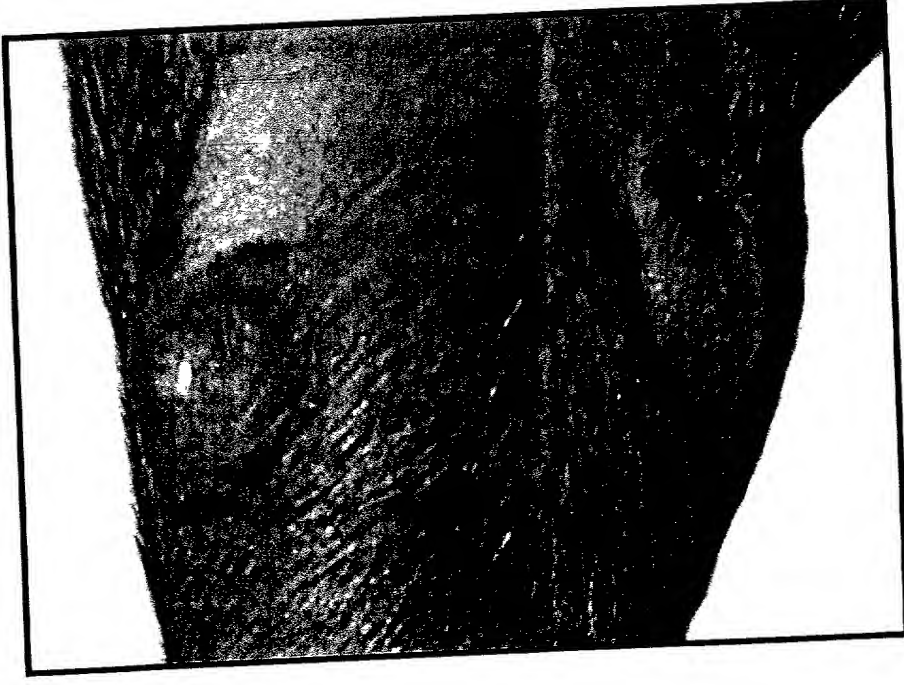
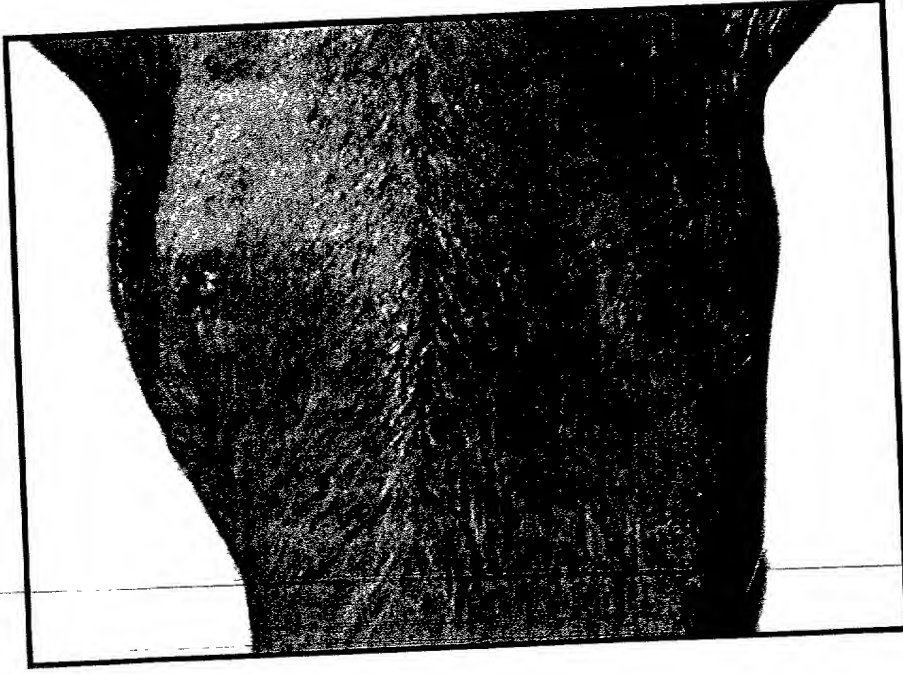


Figure 16A

DOT ESD* 237 E5960



KLA

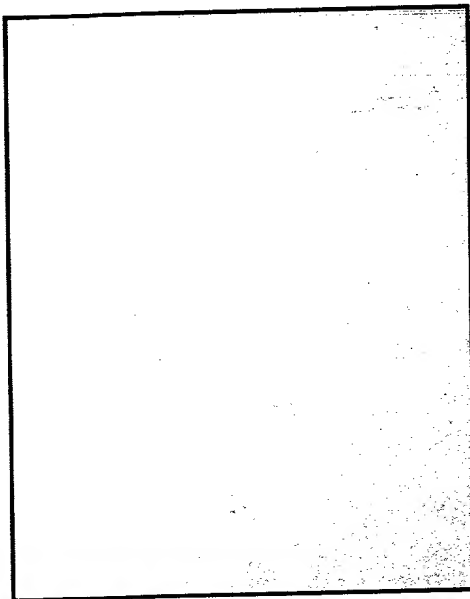
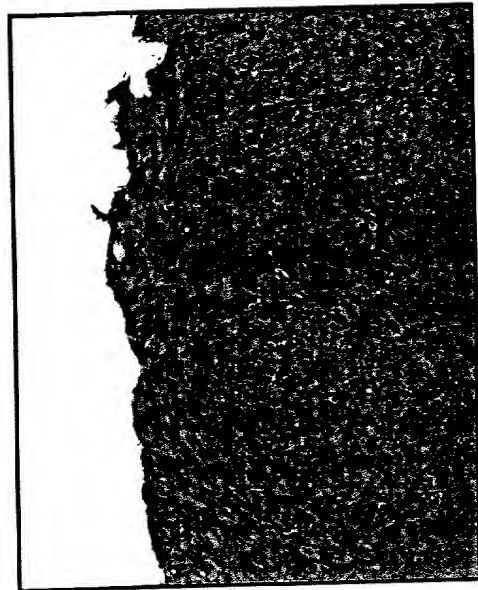


DP1

Figure 16B

00T680" 28T65960

KLA



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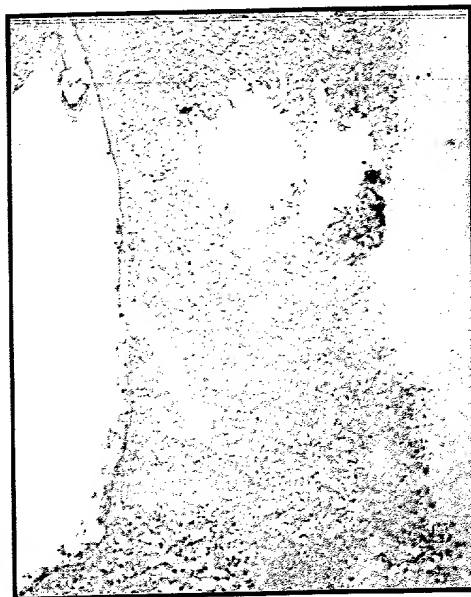


Figure 16C



Figure 16D

CD34⁺/LIN⁻ Stem Cells Are Transduced by a CTP-5-Biotin/Avidin- β -Galactosidase Complex

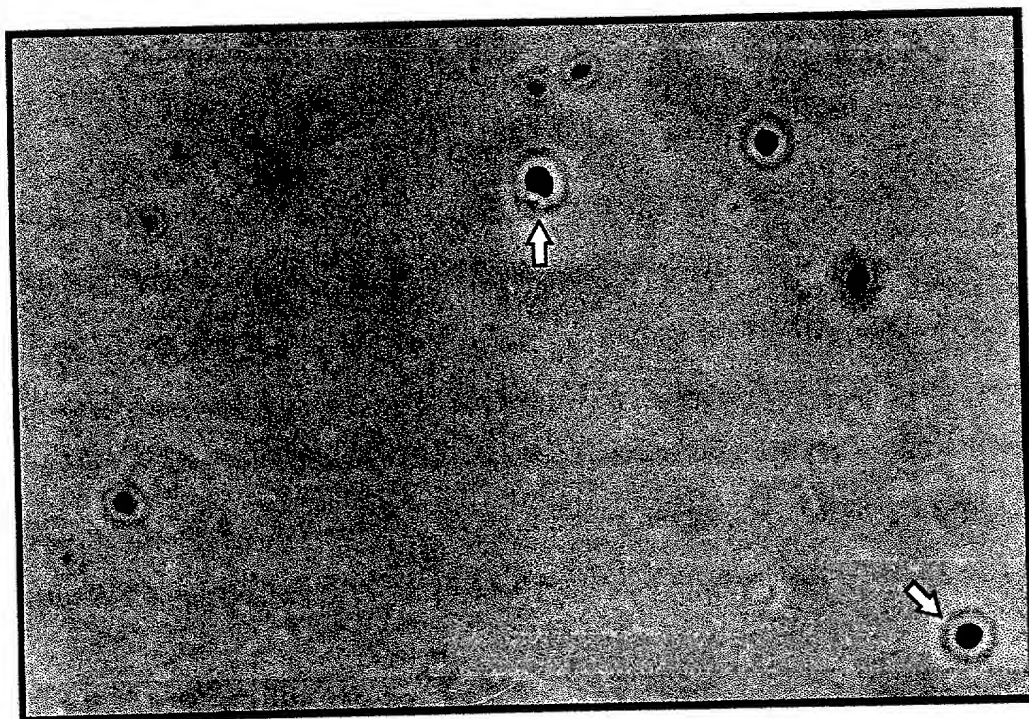


Figure 17

0055312.083100

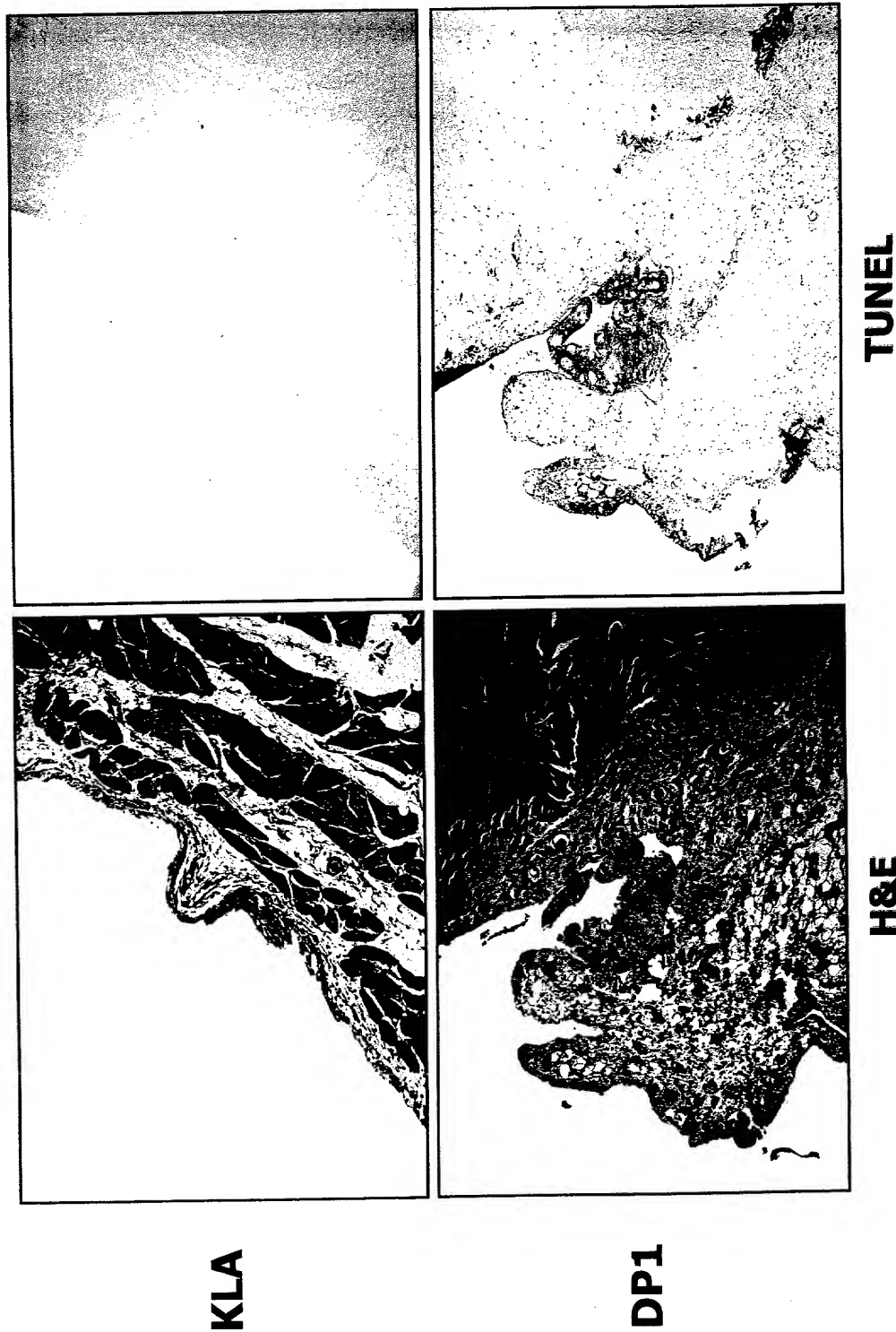


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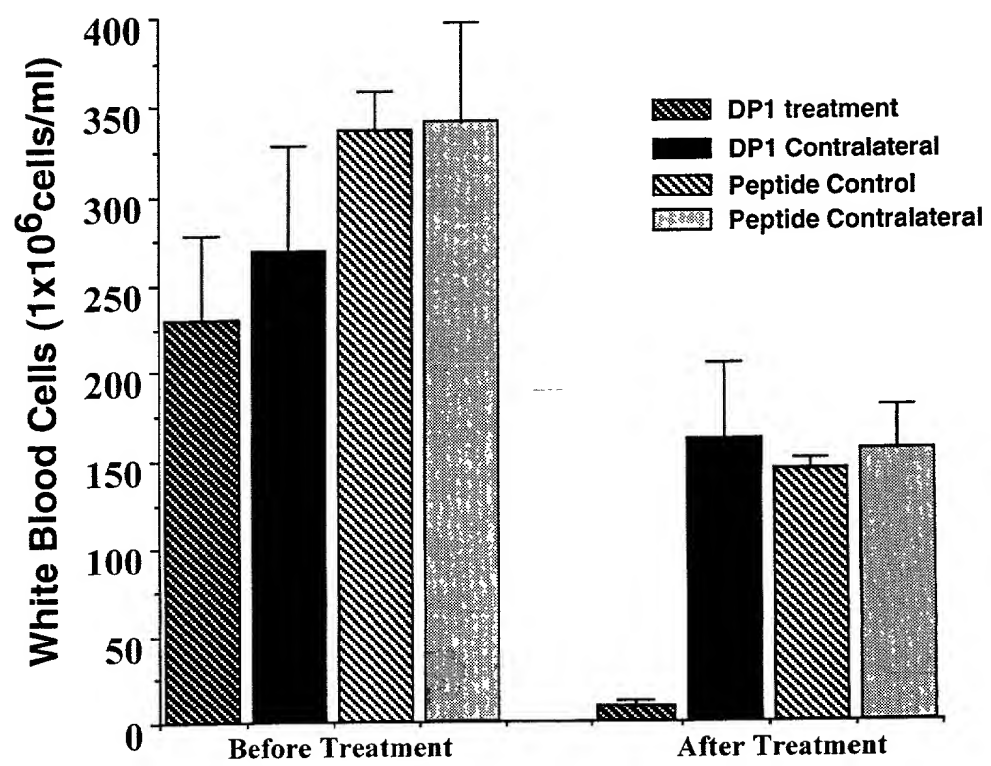


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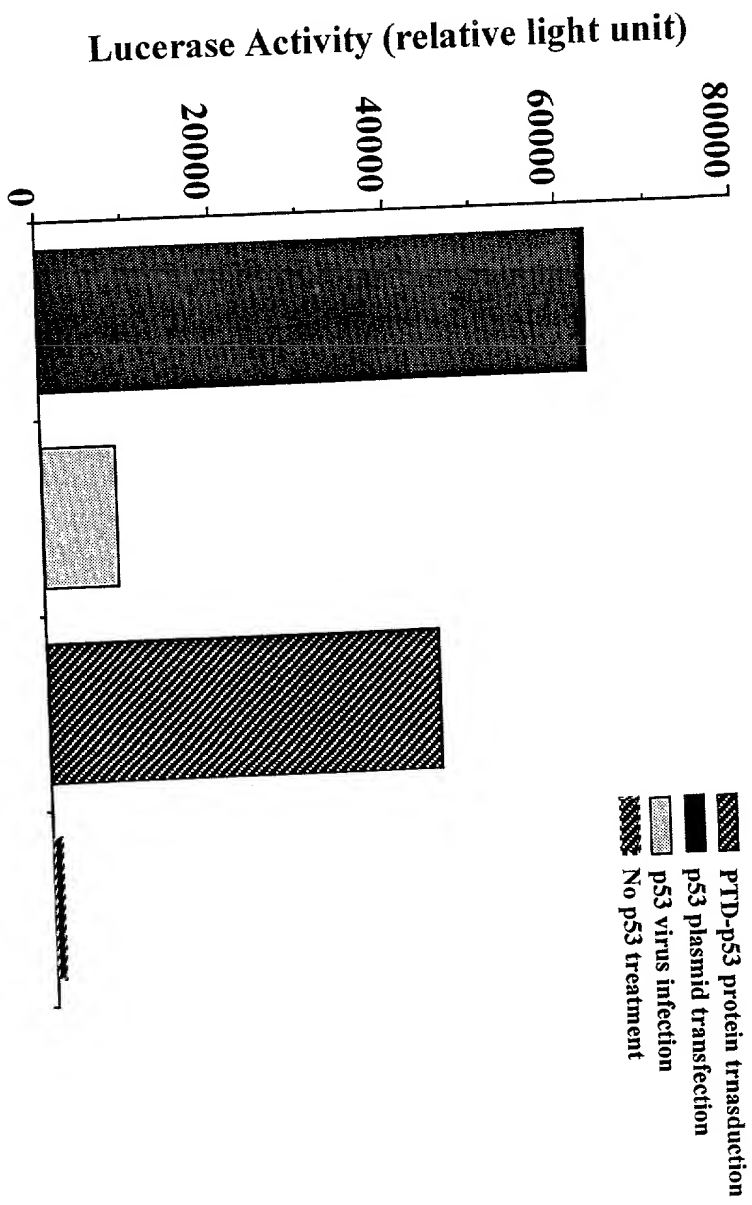


Figure 20

A

B

Figure 21

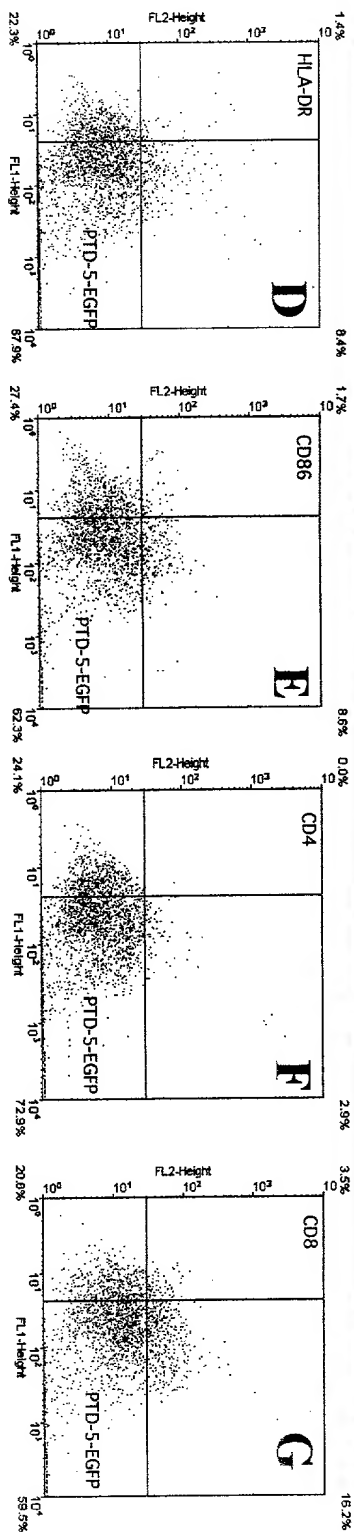
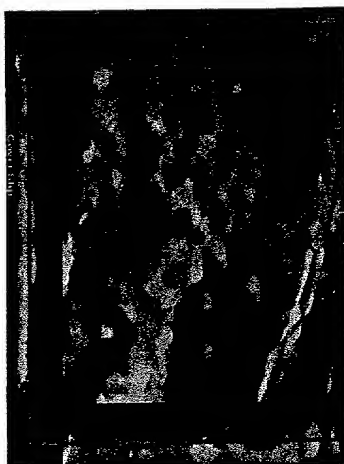
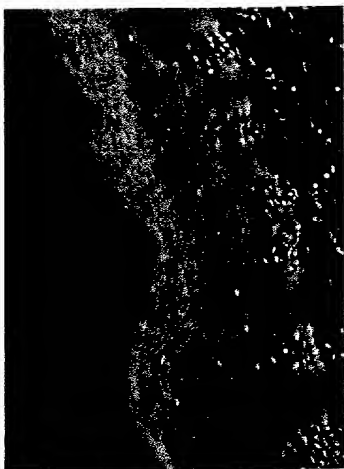


Figure 22

09062013 08:21:00

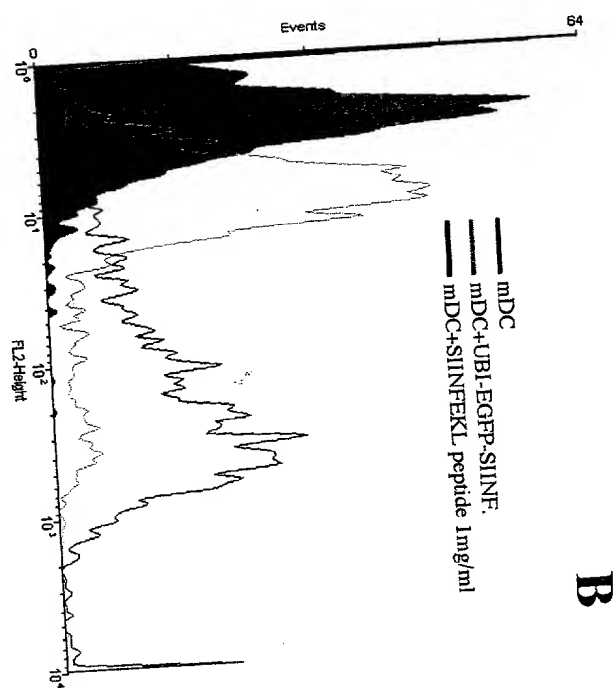
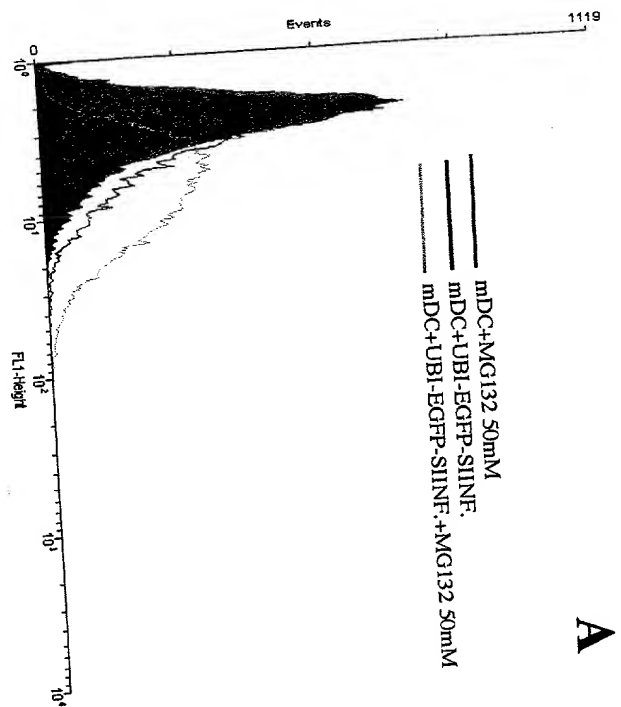
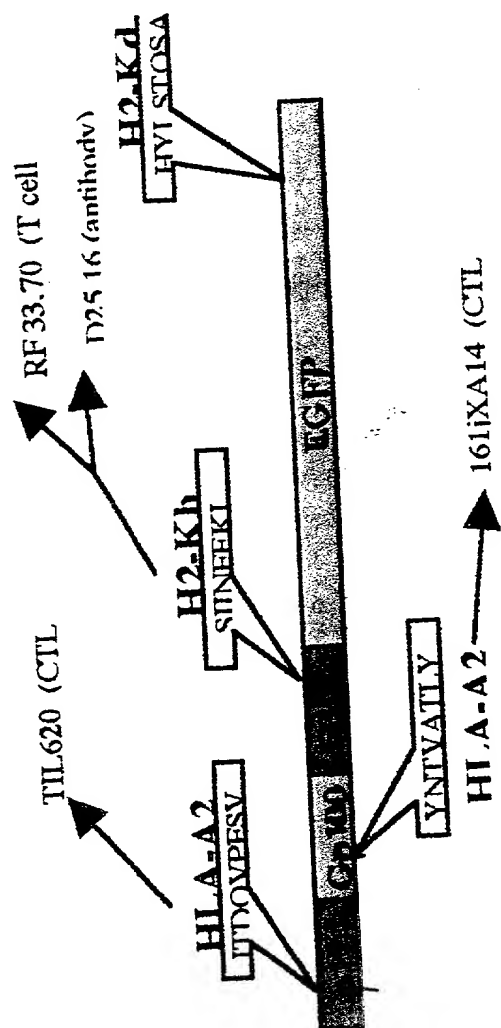


Figure 23

09653182, 0833100



3Epi-EGFP

Figure 24


Applicant: Robbins et al. Filing Date: August 31, 2000
Serial No.: TBA Group Art: TBA
Title of Invention: IDENTIFICATION OF PEPTIDES THAT FACILITATE UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT OF PROTEINS, DNA AND VIRUSES

Assistant Commissioner for Patents
Washington, D.C. 20231

Applicant submits herewith a Sequence Listing in paper and computer readable form for the above-identified application submitted concurrently herewith.

I hereby state that the content of the paper and computer readable copies of the Sequence Listing submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same.

I hereby state that the content of the paper and computer readable copies of the Sequence Listing, submitted herewith in accordance with 37 C.F.R. § 1.82(f), does not include new matter.


Rochelle K. Seide
Patent Office Reg. No. 32,300

Attorney for Applicants
(212) 408-2628

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UPTAKE AND CYTOPLASMIC AND/OR NUCLEAR TRANSPORT
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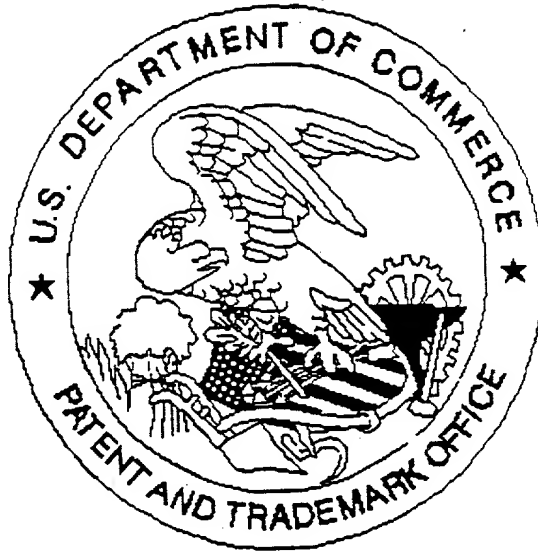
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for scanning. (Document title)

☐ Page(s) _____ of _____ were not present
for scanning. (Document title)

☒ Scanned copy is best available. *Drawings.*

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